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FINAL PROGRESS REPORT FOR AFOSR GRANT # 95-NA-328:

**"Characterization of the Minerals and Iron-Containing
Phases of the Aquifer Material at Columbus AFB"**

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Natural attenuation, defined as physical and chemical processes which decrease contaminant concentrations without human intervention, has been proposed as a cost-effective and reasonable approach for remediation of organic contaminant in aquifers. The USAF Natural Attenuation Study (NATS) is designed to examine the effects of several naturally-occurring processes, including microbial degradation, dilution, and evaporation, on the transport and fate of an organic contaminant plume. This research focuses on one aspect of this process: use of Fe(III) phases in aquifers by select microbes as electron acceptors for the anaerobic oxidation of organic matter.

To accomplish these objectives, the following work was undertaken:

- (1) Thorough characterization of field samples from the NATS site, from the beginning of emplacement through the 1997 field samplings. In addition, source samples collected through the 1999 field season were analyzed as part of this work. Methods included XRD, SEM/EDS, and wet-chemical analysis. Note that this work extended beyond the funding period. All data are included as they are necessary for the conclusions of this work.
- (2) Laboratory investigation of the changes in NATS sediments when reacted with GS-15 or incipient microbial populations. This work was designed to more clearly identify the types of changes that might be expected in the main field experiment.

(3) Investigation of the ability to generate abiotic magnetite, in order to test the validity of using magnetite as a geochemical marker of microbial activity (conducted in part during a summer internship at Tyndall AFB).

This work comprises the thesis of a VIMS graduate student (David C. Powell); the defendable, though not final draft of his thesis is included as the bulk of this final report. This thesis also makes use of other data collected by collaborators on the NATS project to support our findings and justify the selection of samples that were studied in greatest detail; these data also extend to 1999. A summary of the findings of our work, and their significance with reference to specific sections of the attached thesis as appropriate, precedes the thesis draft.

(1) Field Experiment

XRD, SEM/EDS, and acid-extraction of the Fe in the NATS sediments all clearly show that the aquifer is heterogeneous in iron content. This heterogeneity was expected, since similar spatial variabilities over the site region for organic carbon content and hydrocarbon sorption coefficients had been reported by MacIntyre et al. 1998.

This high variability made elucidation of trends in iron mineralogy and content difficult. Nonetheless, consistent decreasing trends in total and amorphous Fe (III) were found in site aquifer materials from the near-field (0-10 meters from the source), primarily in samples closest to the source emplacement (N-4 and N-5). This was expected from the solution chemistry, as the near field was the area that had the highest dissolved hydrocarbon concentrations through time as well as high dissolved inorganic carbon and methane, and elevated aqueous iron (II). This section corresponds to the highest outflow from the source material and suggests that the majority of the iron reduction may be occurring in the source material itself, with the Fe (II) then depositing on the nearby sediments.

Sediment-bound Fe(II) was elevated in the nearest-field sediments (N-5) which also indicates that Fe(II) released upgradient (e.g. in the source region) may sorb to the sediments after transport via groundwater. As such, it is important to note that the dissolved Fe(II) concentrations can only serve as a conservative measure of microbial degradation and reduction of the amount of iron(III) oxide reduced in the affected aquifer material. Additionally, any sorbed Fe(II) may reduce the availability and reactivity of underlying Fe(III) oxides. Therefore, the remaining capacity of an aquifer to degrade hydrocarbons through dissimilatory reduction of Fe(III) oxides cannot be assessed by simply knowing the initial Fe(III) content and estimating loss of these phases via measuring aqueous Fe(II).

(2) Microbial Laboratory Study

The changes in the NATS aquifer material due to dissimilatory reduction of Fe(III)-oxides during oxidation of hydrocarbons also was monitored in the

laboratory. This work was undertaken to avoid the problems of heterogeneity encountered in the field; recall that the high degree of heterogeneity made identifying changes in iron mineralogy difficult. For the laboratory studies, the NATS aquifer materials were homogenized such that each treatment used sediments of the same iron content and mineralogy. Both GS-15 and the incipient microbial population of the NATS study were employed (separately) in this six month experiment. The main findings of this work are as follows:

(1) The surface iron concentrations normalized to silica concentrations (SEM/EDS data) show a slight increase in iron coating the particles. This suggests that solubilized iron has been redeposited on the surface of the particles, perhaps in a form different than for the original iron phases.

(2) Microbial degradation could clearly be documented by the increase in aqueous Fe(II) concentrations and decrease in acetate (for treatments using acetate as the medium). Nonetheless, the amount of the extractable iron in the sediments after reaction did not vary significantly from the abiotic control. As such, it appears that six months is insufficient to show a significant reduction in the iron phases coating the sediments in a laboratory setting. However, this may in part be an artifact of the experimental design. In an aquifer system, any release iron is continually removed via groundwater transport whereas high levels of aqueous iron are achieved in a batch laboratory experiment. These high iron concentrations may suppress further microbial activity and also allow for readsorption of Fe which may "poison" the iron oxide surfaces for further microbial degradation.

(3) Larger surface areas were seen in the GS-15 treatment in comparison to the two naturally occurring treatments. However, this could be due to the transfer of the iron oxy-hydroxide gel on which the GS-15 was cultured. Such material has a very high specific surface area, 600 m²/g for fresh precipitate (Dzombak and Morel, 1990), and so, even a small amount could affect the surface area measured. Furthermore, this amorphous iron is often considered more available for microbial reduction than the relatively more crystalline iron phases present in the sediments. As such, the amorphous iron may be preferentially consumed, thereby reducing the surface area of the system. This could explain the decrease seen in the surface area seen between the sediment only and sediment with GS-15 vials, where the surface area increased between the same vials that were inoculated with the naturally occurring bacteria that were cultured on the iron oxy-hydroxide gel.

In summary, there was considerable consumption of Fe(III)oxides in the CAFB sediment by both GS-15 and naturally-occurring bacteria. The high levels of iron released may have been partially resorbed. The effects of both the high Fe concentrations and this resorption are unknown, but may have caused a decrease in microbially-assisted dissolution of the iron oxides. The majority of the sediment iron oxides were not consumed during the six month incubation

periods. These results indicate that either the capacity for iron reduction has not been reached (and thus longer incubation times are needed) or that the initial capacity diminishes over time due to the build up of iron in the system. While such a build up of aqueous iron is not expected in the field because of groundwater movement, a similar decrease in capacity may occur in the field as iron released due to microbial activity is transported down-field and subsequently resorbed onto the aquifer materials.

(3) Magnetite formation laboratory study.

For this study, parallel abiotic and biotic (containing GS-15) experiments were conducted. For both abiotic and biotic systems, a series of vials were prepared with varying amounts of added Fe(II) from none (control) to 800 ppm Fe; the Fe(III) substrate was an amorphous gel. The goal was to compare the changes in iron mineralogy after one and five months of reaction, and specifically to assess which, if any, are indicative of microbial degradation.

As evidenced by magnetic affinity, optical microscopy and SEM/EDS, magnetite was formed in all the biotic vials and in the abiotic vials to which more than 200 ppm Fe (II) was added. After 1 month, the iron gel in each of the abiotic vials sorbed some of the Fe (II) in solution. After 5 months almost all of the excess Fe (II) introduced into the vials, as well as the Fe (II) produced by the microbes, was sorbed to the iron gel. As sorption of Fe (II) into the iron gel is the first step of magnetite formation, the loss of aqueous Fe (II) suggests the possibility of production of micro-crystalline magnetite inside the ferrihydrite matrix. The small crystallite size of this first formed magnetite is not detectable by powder XRD. In abiotic systems, magnetite did not form to a size or abundance detectable by XRD, even after 5 months. In contrast, the magnetite crystals were larger in the biotic systems, clearly detectable by XRD. Nonetheless, formation of magnetite in the abiotic microcosms demonstrates that magnetite crystallization is a chemical process occurring independent of microbial activity, and magnetite production in itself may not be a sufficient indicator of microbial degradation of hydrocarbons by ferric oxides.

In contrast, siderite, Fe(II)CO_3 , formed in all of the biotic systems except for the 800 ppm Fe (II) addition. The oxidation of acetate by the microbes reduces Fe (III) to Fe (II), increasing the $[\text{Fe (II)}]$, and releases CO_2 in the form of bicarbonate (the system is buffered such that the pH remains near pH 7). Once the solubility product of siderite, $K_{sp}=10^{-10.67}$, is exceeded, siderite precipitates. Therefore, the formation of siderite can be used as an indicator of biological activity in closed systems containing Fe (II). There appears to be a maximum in siderite formation at moderate Fe(II) concentrations (200 ppm Fe (II) addition). One would expect there to be less siderite formation in vials when there is less Fe (II) present, and that siderite formation would increase at higher Fe (II) concentrations. The decrease in siderite, and magnetite, in the 400 ppm Fe (II) additions and the absence of siderite formation in the 800 ppm Fe (II) addition again indicates that the excess Fe (II) sorbed in the ferrihydrite gel decreases microbial oxidation of acetate.

ATTACHMENT:

Draft of David C. Powell's thesis, containing more detailed description of the work including data collected.

1. Abstract

This study investigates the availability of iron-bearing minerals for use by naturally occurring, iron-reducing microbes in the anaerobic oxidation of hydrocarbon groundwater contaminants during a field experiment at Columbus Air Force Base (CAFB), Columbus, Mississippi. It includes the characterization of the CAFB aquifer materials in samples from the field, and from laboratory experiments designed to identify iron mineral microbial reactions that may occur in contaminated aquifers. This work is a part of the Natural Attenuation Study (NATS) conducted by the USAF to determine the viability of natural attenuation as a cost effective and reasonable remediation technique for an organic contaminant plume.

Several instrumental and chemical analysis techniques were used to study the bulk mineralogy and iron phases present in the CAFB aquifer material and in laboratory samples. Powder x-ray diffraction (XRD) was used to determine the minerals present in the aquifer, aid in the identification of amorphous iron phases and identify secondary iron phases formed in the laboratory studies. Ferrous and ferric iron were extracted from solid phases with HCl extractions. Total extracted iron was analyzed by inductively coupled plasma, atomic emission spectrophotometry (ICP/AES) and by flame atomic absorption (FAA). Ferrous iron concentration, released during the HCl extraction, was determined by complexing with ferrozine, followed by UV/Vis spectrophotometric analysis. In addition, scanning electron microscopy coupled with energy dispersive x-ray analyses (SEM/EDS) was done to obtain information on average iron content of mineral coatings and spatial variability. SEM images also provided topological information about the sediment and secondary minerals. Specific surface area analysis (SAA) was determined by the B.E.T. nitrogen gas absorption method.

2. Introduction and Background:

This work investigates the role of iron bearing minerals in natural attenuation of organic contaminants in aquifers. Natural attenuation, defined as any naturally-occurring process that decreases the concentration of contaminants in groundwater, has been proposed as viable, low cost, technique for remediation of hydrocarbons in contaminated aquifers. For natural attenuation to be considered feasible, biodegradation must be shown to be taking place and the maximum extent of the dissolved contaminant plume migration must be estimated (Borden et al., 1997). The Natural Attenuation Study (NATS), conducted by the United States Air Force (USAF) Armstrong Laboratory, Environmental Quality Laboratory (AL/EQL), is designed to examine the effects of several naturally-occurring processes, including microbial degradation, dilution, and evaporation, on the transport and fate of an organic contaminant plume. As the goal of remediation design is to reduce the contaminant concentration in the aquifer to a level that no longer threatens human health or the environment (Nyer, 1997), natural attenuation may be the best choice remediation design in areas where other remediation techniques will cause significant environmental damage (Boggs et al., 1993).

Uncontrolled release of hydrocarbons to subsurface systems is widespread. Leaking underground storage tanks, improper disposal of hazardous materials in landfills and excessive use of agricultural chemicals are all important sources of contamination (Mihelcic et al., 1995). In most cases, contaminants dissolved in the water column are of primary importance. NATS focuses on contamination by petroleum derived fuels, especially the primary dissolved components known as BTEX (benzene, toluene, ethylbenzene and xylene). BTEX makes up less than 20 percent of fuel mixtures like jet fuel, gasoline and diesel fuel, but accounts for 82 percent and 98 percent of the dissolved compounds from fresh JP-4 and gasoline, respectively (Wiedemeier, et al., 1996).

Contaminant plume movement is caused by advection and dispersion of the water-borne contaminants through the aquifer. Factors controlling the attenuation of groundwater contaminants include dilution, retardation, precipitation, ion exchange, and abiotic and microbial degradation (Nyer, 1997, Wiedemeier et al., 1996). Of these processes, only the degradation processes (i.e. oxidation) are capable of transforming the hydrocarbons into innocuous by-products, and typically, microbial degradation is much faster than abiotic.

Upon introduction of hydrocarbons into an aquifer, zones of oxidation of the contaminant plume form by sequential consumption of terminal oxidizing agents (e.g. O_2 , NO_3^- , and Fe (III)). An idealized distribution of terminal oxidation processes (TOP) spreading from a source of hydrocarbon contaminants would include, in order of increasing redox potential, methanogenic, sulfate-reducing, Fe (III)-reducing, nitrate and Mn(IV)-reducing, and oxic processes (Lovley et al.,

1994). The existence of each of these zones requires availability of the appropriate electron acceptor within the aquifer.

The most common evidence of natural attenuation of hydrocarbons by microbial processes includes: (1) documented loss of contaminants at the field site, (2) chemical and geochemical data, including increased concentration of intermediates and metabolites of anaerobic metabolism of BTEX, and concomitant decreased concentration of terminal electron acceptors in the aquifer, and (3) laboratory culture studies, showing microbial degradation (Wiedemeier et al., 1996; Wilson and Madsen, 1996). In addition, there are several other parameters that provide evidence for bioremediation. Differences between the $\delta^{13}\text{C}$ values of hydrocarbons and indigenous carbon sources (e.g. plant matter, soil carbonates) can be exploited to trace the origins of metabolic end products (Conrad et al., 1997). Also, the concentration of dissolved H_2 has been shown to correspond to the reduction-oxidation (redox) potential and is used to determine which TOP predominate at specific locations in an aquifer (Lovley et al., 1994). Finally, in situ molecular biology measurements using gene probes during NATS have shown directly the redox activity of indigenous microorganisms in oxidative of the source hydrocarbons during this experiment (Stapleton et al., 2000).

The observed distribution of microbial activity in an aquifer depends, in part, on the availability of nutrients that, in turn, is controlled primarily by the physical and chemical heterogeneity. Low conductivity inclusions in an aquifer may greatly enhance microbial degradation of hydrocarbons due to prolonged availability of substrate (Murphy et al., 1997). Mobile colloids enhance groundwater contaminant transport by acting as carriers for contaminants adsorbed to their surfaces. Mobile bacteria may also act as carriers by transporting contaminants sorbed to their cell walls (Kim and Corapcioglu, 1996).

Unlike previously known iron-reducing bacteria that only partially breakdown hydrocarbons through a fermentation pathway, *Geobacter metallireducens*, is the first microorganism known to completely, anaerobically oxidize aromatic hydrocarbons to CO_2 using Fe (III) as a terminal electron receptor (Lovley and Phillips, 1988). It also was the first pure culture capable of anaerobically oxidizing toluene, a common component of petroleum products such as gasoline and jet fuel (Lovley and Phillips, 1988; Lovley and Lonergan, 1990). More recently, other bacteria have been cultured that use Fe (III) to completely oxidize hydrocarbons. For example, *Shewanella alga* strain BrY grows in a medium with synthetic goethite as the only oxidant, reducing the Fe (III) by 8-18% (Roden and Zachara, 1996) and *Pelobacter carbinolicus* is able to use both Fe (III) and S^0 as terminal oxidants (Lovley et al., 1995). The evidence of such iron-reducing bacteria in the laboratory warrants further study of the possibility that such processes occur in the natural environment.

There is concern that highly crystalline iron(III) oxides may not be available for microbial remediation of hydrocarbons. Some studies of aquatic sediments and submerged soil show that crystalline iron(III) oxides like goethite($\alpha\text{-FeOOH}$) and hematite($\alpha\text{-Fe}_2\text{O}_3$) may not be reducible by microorganisms, but amorphous and poorly crystalline iron(III) oxides are readily

reduced (Phillips et al., 1993; Lovley and Phillips, 1987). Other studies have shown that Fe (III)-reducing bacteria may be able to survive and produce significant quantities of Fe (II) in anaerobic soils and subsurface environments where crystalline iron(III) oxides such as goethite are the dominant forms of Fe (III) (Roden and Zachara, 1996). Difference in utilization may be a function of the naturally-occurring consortium of bacteria. Reduction of even small amounts of iron(III) oxides can produce significant changes in the geochemistry of an aquifer. Reduction can release soluble Fe (II) and other adsorbed species (e.g. phosphates and trace metals), and provide a reactive Fe (II) surface capable of participating in secondary redox or mineral-forming reactions (Roden and Zachara, 1996).

This study was designed to investigate changes in the iron-bearing phases in the Columbus AFB aquifer associated with hydrocarbon degradation the NATS experiment. The site aquifer has low concentrations of dissolved sulfate and nitrate and little solid manganese oxides, so microbially-mediated iron(III) reduction of the emplaced hydrocarbons is expected to be the primary oxidant once the oxygen has been depleted. Specifically, this iron mineralogy portion of the NATS experiment was designed to characterize changes in the Fe (III)-bearing phases due to microbial degradation and to assess the availability of these phases to support degradation processes.

2.1. Overview of NATS Project

Natural attenuation is a term apparently first used by Barker et al. (1987) to describe the set of physical and chemical processes occurring in aquifers that diminish contaminant concentrations over time without human intervention. Quantification of natural attenuation at a contamination site requires definition of initial (source) conditions and measurement of contaminant transport and fate during the evolution of the contaminant plume. The field experiment called the Natural Attenuation Study (NATS) was initiated in November 1995 in an unconfined aquifer at Columbus AFB (Fig. 1).

The shallow unconfined aquifer in which NATS was conducted consists of an alluvial terrace deposit averaging 11 meters in thickness. The aquifer is composed of poorly sorted to well sorted sandy gravel and gravely sand with minor amount of silt and clay. Marine sediment of the Eutaw Formation consisting fine sand silt and clay acts as an aquitard below this alluvial aquifer. A detailed site description of the site and its geology is given in Boggs et al. (1992).

The hydrology of the aquifer is well known from previous studies (Boggs et al. 1993; MacIntyre et al. 1993). The local groundwater has low organic carbon, nitrogen, phosphorous and sulfate concentrations. Mean dissolved concentrations for sulfate, potassium and nitrate-nitrite are 2.0 mg/L, 0.6 mg/L and 1.5 mg/L, respectively (Boggs et al., 1993). Additionally, iron oxide coatings on the aquifer material range from 2.0 to 3.9 percent by weight for particle diameters less than 2mm, and from 0.5 to 0.8 percent for particles greater than

2mm (Boggs et al., 1993). Aquifer sediment organic carbon concentrations are quite low, ranging from 0.02 to 0.06 percent (MacIntyre et al., 1991).

To initiate the NATS experiment, a large hydrocarbon, non-aqueous phase liquid (NAPL) mass was emplaced in the saturated zone of the aquifer. The leaching, transport and geochemical fate of the NAPL components were then observed over space and time to measure their attenuation by processes including microbial degradation and dilution. Agencies cooperating in NATS were the USAF AL/EQC-OL, Tennessee Valley Authority, College of William and Mary, University of Tennessee, and Florida State University. The NATS experiment had several research objectives that provided quantitative information to support implementation of natural attenuation as a remediation action at groundwater contamination sites throughout the nation. The need for better understanding of natural attenuation processes to support remediation actions is made apparent from conclusions of the Groundwater Cleanup Alternatives Committee report (NAS, 1994). This report describes the unfeasibility of conventional active groundwater cleanup processes for most contamination situations. Based on subjective and qualitative thermodynamic analysis of remediation operations in contaminated aquifers, the report concludes that existing active remediation actions are not effective or economically feasible at most sites, and probably cause more damage to the world environment than would occur without active remediation.

A satisfactory site remediation by natural attenuation is indicated by maintenance of steady-state contaminant concentrations at the downgradient edge of the plume. These concentrations must be below the applicable water quality standards for the site. Natural attenuation is considered a successful remediation action if this condition is realized within the site land parcel such that, no groundwater outside site borders has contaminant concentrations exceeding water quality standards, and no surface water bodies (including lakes, rivers and estuaries) are contaminated by groundwater seepage across the sediment-water interface.

In the NATS experiment, initial conditions of source location, amount, composition and emplacement time, and of hydrological parameters such as hydraulic heads and conductivities were established. With this information, the experiment results can be used to demonstrate the attainment of a natural attenuation plume condition, and to address the feasibility of natural attenuation actions at other sites, as specified in the objectives (see Section 6.1.1). Observations at existing contamination sites can not provide comparable information on natural attenuation processes in aquifers, because they have poorly known initial conditions and histories, and are thus unsuited for obtaining the quantitative information on hydrocarbon fate and transport. However, such sites can show qualitatively that natural attenuation is occurring, and their investigation is a necessary component in demonstrating the practicality of natural attenuation as a remediation action.

2.1.1. Objectives of the NATS program

The primary objectives of NATS were to show the development from known initial conditions of a steady state plume distribution maintained by natural attenuation processes, and to provide information on the chemical, geomicrobiological and transport processes that are components of natural attenuation of hydrocarbon contaminants in aquifer environments. This research provides a scientific framework for use by managers in decisions regarding the implementation of natural attenuation at contamination sites in order to protect groundwater supplies and surface water bodies.

Several parameter measurement programs were included in the NATS field experiment that contributed to achievement of these objectives. The concentrations of hydrocarbons, oxygen, ferrous iron, inorganic carbon, methane and ^{13}C isotope abundances in groundwater samples were measured. Spatial and temporal distributions of these analytes are used to demonstrate natural attenuation of the hydrocarbons leaching from the source region. Quantification of natural attenuation of the emplaced NAPL also required core sampling of the source and downgradient regions to obtain information for a mass balance of carbon. This was the first field experiment to investigate natural attenuation of a large NAPL in an aquifer, and the initial conditions for the experiment were chosen with the intent of attaining a steady state plume within the test site boundaries.

The NATS experimental design allowed investigation of the role of ferric iron oxide minerals commonly occurring in oxygenated aquifers in oxidation (degradation) of hydrocarbon contaminants after oxygen has been locally depleted by aerobic degradation. This information is needed to determine whether the aquifer has an oxidizing capacity in excess of that provided by the dissolved oxygen in ground water. In sites where oxidation by iron oxides is significant, existing fate and transport prediction models for hydrocarbons in groundwater, such as BIOPLUME II (Rifai, 1989) that treat only oxidation by dissolved oxygen are inadequate. Since hydrocarbon oxidation by iron oxides is found appreciable in NATS, the resulting data set is now being used to develop a fate and transport model for hydrocarbon fuels that includes this oxidation (Widdowson, 2000). The resultant model may then be applied to other site situations where iron oxides are present and dissolved oxygen is depleted. In order to support the evaluation of the contribution of iron oxides to hydrocarbon degradation by the activities of the iron bacteria (the subject of this thesis), aquifer material mineralogy, chemical composition, and oxidation capacities were determined by various members of the NATS research team.

2.1.2. NATS Experiment Description and Aqueous Chemistry

The first stage of the NATS experiment was emplacement of the hydrocarbon NAPL mixture coated on native sand in a steel sheetpile lined

source trench (Figure 2) from which water and sediment were removed. Pumping nearby wells to dewater the trench region started November 1, 1995 and continued through November 11, 1995. with removed. Excavation of 1040 m³ of sediment and shoring of the sheetpile cell in the trench was done November 4-9, 1995. Soil and sediment excavated between ground surface and the water table were stockpiled separately from sediment below the water table. Mixing and placement of the hydrocarbon coated source material was done over seven hours beginning at noon November 10, 1995. Two cement mixers were used to mix the sand fill with the hydrocarbon solution and aqueous bromide solutions. Eight 3.75 m³ batches were prepared and placed in the bottom of the trench. Composition and amounts of hydrocarbons added, based on masses added to mixers, are given in Table 1. Four 80 ml samples of the source material were collected from each mixer during mixture placement and analyzed to confirm these amounts. The source zone was cored and analyzed for hydrocarbons within a month after the start of the experiment in order to prove that loss of hydrocarbons due to vaporization during emplacement was not significant.

A bromide solution was prepared on-site using groundwater obtained from the dewatering. Two batches were prepared, ranging from 120 to 140 mg/L with an average of 128 mg/L, for a total of 5300L of solution. Approximately one eighth of this solution was added to each of the eight sand - hydrocarbon batches in the final stage of the mixing process to serve as an inert (conservative) indicator of plume movement.

Table 1: Composition of the hydrocarbon mixture (NAPL) emplaced in the source trench.

Compound	Mass (kg)	Mass Fraction of total	Mole Fraction of total	Solubility	Mixture Solubility
Decane	856.42	0.7463	0.6878	0.009	0.006
Benzene	0.577	0.0005	0.0008	1760	1.6
Toluene	73.02	0.0636	0.0906	515	54
Ethylbenzene	71.84	0.0626	0.0773	152	14
p-xylene	72.65	0.0633	0.0782	198	18
Naphthalene	73.11	0.0637	0.0652	100	12

On completion of source placement, elevations across the top and bottom of the source, as well as the spatial coordinates of the corners of the source were surveyed. About 0.5 m of backfill was then poured over the source material to minimize hydrocarbon (HC) vapor losses. The next morning the trench was backfilled to about 3 m below grade and dewatering terminated. Just prior to cessation of dewatering, water samples were collected from each of the three operating dewatering wells. Backfill of the trench and removal of shoring was done on November 14, 1995. Sheetpiling was removed by pulling vertically on

Nov.30- Dec1, thereby commencing the NATS experiment by starting aquifer flow through the source zone .

2.2. Summary of groundwater solute temporal and spatial distributions in NATS

Initial analysis of geochemical changes during NATS was done using solute concentrations for all wells in each of seven zones that were defined by distance normal to the long axis of the source box (Figure 3). Each zone extends laterally 12.5 meters from of a line normal to the long axis of the source with origin at the source center. Each well shown in Figure 3 contained a multilevel sampler with 20 to 30 separate sampling points spaced 0.38 meters apart in the vertical direction, in general spanning the thickness of the unconfined aquifer. Details of multilevel sampler construction are given in Boggs et al. (1988).

The monitoring wells (identified by well numbers shown in Figure 3) in each zone were:

- 10 to 0 meter zone: well numbers 57- 59 (upgradient of source)
- 0 to 5 meter zone: well numbers 10-14, 38, 43, 209, 295-298
- 5 to 10 meter zone: well numbers 15-21, 44-46, 210-214, 299, 300, 302
- 10 to 15 meter zone : well numbers 22-27, 49, 50, 303-305
- 15 to 20 meter zone: well numbers 28-32, 52, 65-69
- 20 to 25 meter zone : well numbers 73-79
- 25 to 30 meter zone : 89-92

Concentrations were averaged over all well point samples for all wells in each zone, with the process repeated for each sampling time ("snapshot"). These averages provide a measure of spatial and temporal distributions that can be used to determine geochemical changes occurring in the experiment (see Appendix 12.1). The limitations involved in use of these average values as measures of geochemical change are that averaging obscures heterogeneity at lengths less than the zone size, and that well point spatial coverage within each zone may not allow determinations of "representative" or "true" values for the zones. Earlier experiments at the NATS site have shown heterogeneity at lengths less than 5m (MacIntyre et al, 1993). Quantification of the effect of sample spatial density on the averages reported was not possible as it would require installation of many more wells, which was precluded by cost and site disturbance considerations.

Nonetheless, the zone concentrations results can be used to examine changes in parameters that are indicators of hydrocarbon degradation. Also correlation among parameters can be used to identify biogeochemical processes occurring in natural attenuation of the source. Interpretation of the analytical results is ongoing by NATS collaborators' involved in these various components of the NATS project. However, an overview of the geochemical changes

relevant to microbial use of iron oxides during the hydrocarbon degradation can be extracted from these collaborators data (Appendix 12.1). Primary among these considerations is the relative transport of the bromide and hydrocarbon (HC) pulses, and the appearance of ferrous iron and methane pulses. The bromide pulse can be followed spatially and temporally throughout the entire monitored aquifer extending out approximately 30 meters from the source, where the HC pulse primarily extends only 10 m from the source with a trivial amount shown to have extended 15 meters. The methane and Fe (II) pulses were maximized in the 0-5 meter zone from the source. These aqueous results clearly show that the hydrocarbons are being degraded and that conditions are at least reducing enough for iron oxide reduction to have taken place. Therefore, changes in iron mineralogy may be noticeable extending up to 10 meters from the source material.

2.3. Overview of this research on the role of iron in NATS.

2.3.1. Field Experiment

The NATS experiment was designed to create redox conditions that might produce changes in the iron-bearing phases in the Columbus AFB aquifer. The amount of dissolved Fe(II) released to groundwater during the experiment indicates that iron minerals were reduced in region near the source by concomitant oxidation of hydrocarbons leached from the NAPL source by groundwater. The site has low concentrations of sulfate, nitrate and manganese, so microbially-mediated iron(III) oxidation of the emplaced hydrocarbons will be the primary redox process once molecular oxygen has been depleted. Specifically, this study was designed to characterize possible changes in the Fe (III)-bearing phases due to microbial degradation at selected core locations downfield from the NAPL source. It was also intend to provide data augment data collected our NATS collaborators in assessing the capability of iron oxide phases to support degradation processes. This characterization was done primarily by determining total iron, amorphous iron, and Fe (II) concentrations in extracts of site geological solids using selective extractants. Also the iron-bearing phases were examined by powder x-ray diffraction, and surface iron concentrations and morphology were assessed by scanning electron microscopy/energy dispersive spectroscopy (SEM/EDS).

2.3.2. Laboratory Experiments with Iron minerals and Aquifer Materials

Laboratory experiments were conducted to explore reactions of site aquifer materials and iron-containing minerals with hydrocarbons occurring under controlled environments. The first experiment was designed to give possible indications of what may be observed in the NATS field situation. The second

experiment was designed to examine formation of secondary minerals (e.g. magnetite) during the consumption of hydrocarbons as a function of dissolved Fe(II) concentration. Results of these experiments are of qualitative value, but cannot be extrapolated quantitatively to make field site concentration predictions due to differences between the field and laboratory situations which include:

- 1) There is a high spatial heterogeneity of the aquifer sediment properties that can produce microzones of oxic and anoxic degradation that are separated by short distances. This situation does not apply to laboratory studies of small homogenized samples .
- 2) There were some hydrocarbons in the field NAPL that were not used in laboratory experiments, and hydrocarbon concentration did not vary spatially in the laboratory batch experiments .
- 3) The field is a flow through system, that could not be replicated in the laboratory. Aqueous concentrations of HC and degradation products including Fe (II) will be "flushed" from the aquifer, unlike in the laboratory. Thus, concentrations of reactants obtainable in the laboratory batch systems are much higher than in the field, so that reactions observed in the laboratory may not be important at field sites.

3. Methods of Analysis of Aquifer Materials

3.1. Details of Analytical Methods

A suite of analytical methods was employed in this study in order to assess the chemical and physical changes in aquifer materials coinciding with microbial oxidation of organic materials. These include powder x-ray diffraction (XRD), specific surface area (SSA) analysis by the B.E.T. nitrogen gas absorption method, scanning electron microscopy coupled with energy dispersive x-ray spectroscopy (SEM/EDS), gas chromatography, selective iron extractions, total iron analyses by flame atomic absorption (FAA) or inductively coupled plasma spectrophotometry (ICP), and Fe (II) analyses by complexation with ferrozine and analyses on a UV/Vis spectrophotometer.

X-ray diffraction (XRD):

Approximately 0.5 g of sample was ground with a corundum mortar and pestle to a fine powder, about the consistency of corn starch, and loaded onto sample holders fitted with a zero background quartz plate. XRD analyses were done on the < 0.45 mm size fraction of all samples and on the fine fraction (< 15 um, separated by centrifugation methods) of a few samples. XRD data generally were collected on a Scintag X₂ ADS system using an iron anode, a Peltier (solid state) detector over a 4 to 100° 2 θ range in 0.02° steps for 8 seconds/step. Background subtraction was performed using a manual three point spline method and peak identification was based on comparison to an ICCD(II) file. The expected pattern for amorphous iron was taken from Schwertmann et al.

(1982). Additionally, a small subset of samples were scanned with finer resolution over the primary iron oxide peaks to discern changes in these phases; data were collected over $2\theta \approx 43-47^\circ$, for 1 to 2 days which improved signal-to-noise although the data were still "noisy".

Specific surface area (SSA):

Specific surface area (SSA) is the mass-normalized surface area of a sample, expressed as m^2/g ; small particles and porous particles tend to have higher SSA. SSA was determined by the B.E.T. method using nitrogen gas absorption. Approximately 0.1 g sub-samples of freeze-dried sediment were prepared in the Micromeritics flow-prep station by passing nitrogen gas through the sample while heating at 102°C for at least two hours. The sample was then capped, cooled and accurately weighed before the measurement of nitrogen adsorption using a Micromeritics Gemini 2375, balanced tube instrument. SSA was based on a 5 point nitrogen adsorption curve using the BET equation (Gregg and Singh, 1982).

Scanning electron microscopy / energy dispersive x-ray spectroscopy (SEM/EDS):

SEM /EDS with an Amray scanning electron microscope and attached IXRF EDS was used to provide morphological and chemical data on the iron phases. The freeze-dried sediment sample was dispersed over an aluminum stub, attached by a glue tab adhesive, and the sample then coated with 0.2-0.3 nm of gold-palladium to minimize charging during imaging. An SEM image was acquired and 15 individual particles were randomly "tagged" for chemical analysis by EDS. Spectral analysis data was collected for each of the tagged particles, and the iron to silica ratio of the particle was determined using an internal calibration.

Gas Chromatography (GC) analysis of acetate:

Concentrations of acetate were quantitatively determined on a Varian 100 gas chromatograph. Acetate samples were acidified by adjusting the pH of the sample to at least 1 pH unit below the pK_a of acetate, 4.98. The polyethylene glycol ester column used for volatile fatty acid analysis can be damaged by repeated exposure to low pH solutions, so care was taken to not acidify the solution to below pH 3. The injection port and flame ionization detector were heated to 220°C , the column was kept at a constant 180°C , and the helium carrier was $\sim 8\text{cps}$. Note that although one set of laboratory experiments used toluene as the hydrocarbon source, toluene concentrations could not be accurately determined by GC or other methods because of the potential loss of this volatile aromatic hydrocarbon to the head space in these experiments.

Selective Extraction of Iron from aquifer sediments :

The fraction of iron present in amorphous phases was determined by comparison of selective extraction for amorphous iron phases and complete extraction of iron phases. Additionally, the distribution of iron between redox states -- ferrous, Fe(II), and ferric, Fe(III) in the amorphous phases was calculated (see details below). The total iron extractions do not preserve the initial redox state of the extracted iron, and thus the total Fe(II)/Fe(III) ratio of the sediment iron oxide phases cannot be determined.

Amorphous iron, both Fe (II) and Fe (III), was extracted using 1 N HCl. Specifically, 0.25 g sediment and 20 ml N₂-sparged 1N HCl were sealed in centrifuge tubes with the head space filled with N₂. The samples were then periodically agitated in a water bath for 30 minutes at 80°C (modified from Chao and Zhou, 1983). Alternatively, for some of the extractions, the sediments and acid solutions were allowed to equilibrate in an anaerobic glovebox before the reaction. The sediment and acid were then sealed in serum vials with Teflon coated septum, brought out into the laboratory, and placed in the water bath. By performing these extractions under N₂, the redox distribution of amorphous Fe is preserved and thus can be determined analytically (see below).

Two methods of total Fe extraction were evaluated for this study using a subset of the aquifer samples. Because Fe(II) comprises a small fraction of the total Fe in oxidizing aquifers, total Fe is similar to the total ferric iron concentration. Each extraction procedure was replicated at least twice for ≈15-20 samples. For the first procedure, total Fe (III) oxides were extracted by reductive dissolution using the citrate-dithionite-bicarbonate (CDB) method (Heron et al., 1994); already reduced Fe(II) in the sediments is also mobilized during this extraction via direct dissolution. For the extraction, 0.25 g sediment, 20 ml 0.3 M Na citrate and 2 ml 1.0 M NaHCO₃ were mixed in a centrifuge tube and heated in a water bath to 80°C; next 0.5g Na₂S₂O₄ was added. The sample was returned to the bath for at least 15 minutes with periodic mixing. After which, the samples were centrifuges and the supernatant was removed for iron analyses. This procedure was repeated at least three times with each sample, combining extracts, to obtain quantitative results of Fe (III). The second procedure for removing total Fe was a single extraction of 0.25 g sediment with 20 ml of 5 N HCl for two weeks at room temperature (Heron et al., 1994); hereagain, while this extraction procedure is designed to attack Fe(III) oxides, Fe(II) phases are also dissolved. The 5N HCl method reproducibly yielded the most extractable iron, as determined by XRD analysis and FAA analysis, and was used for all subsequent extractions.

Iron Analysis:

The supernatants from the various extraction procedures were analyzed to determine the amount of Fe (II) and Fe (III) released. Fe (II) was determined by complexing 2 ml of extract supernatant with 2 ml of 0.004 M ferrozine

buffered with 0.05M N-[2-Hydroxyethyl]piperazine-N'-2[ethanesulfonic acid] (HEPES) and analyzing on a Shimadzu UV/Vis spectrophotometer at 562 nm. Fe (II) was only analyzed for the 1N HCl extractions (i.e. only for amorphous iron). The CDB extraction process reduces all Fe (III) to Fe (II), thereby not allowing distinction of Fe(II) and Fe(III), and the 5N HCl extract would overwhelm the buffering capacity of the ferrozine solution. Total iron was determined for all extractions (1 N HCl under N₂; CDB; and 5N HCl) by ICP (using a Thermo Jarrel Ash TraceScan) or by FAA (using a Perkin Elmer AAnalyst 800). Amorphous Fe (III) is calculated as the difference between Fe (II) and total iron concentrations determined in the 1N HCl extracts.

3.2 Evaluation of analytical data on aquifer material samples

Field Samples

Aquifer material core samples collected during NATS were analyzed by several methods in order to identify possible changes in the iron phases due to dissimilatory iron-reduction by anaerobes. The aquifer sediment samples were characterized by XRD, acid extraction of iron in various forms, SEM/EDS, and SSA analysis. While each method provides important information, results from several methods of analysis were combined to present stronger evidence of the possible changes in the iron phases of the aquifer material. A key approach employed to minimize grain size effects on data for samples from this heterogeneous aquifer was to conduct all the analyses on the <0.45 mm diameter sized particles obtained by sieving through a 0.45 mm mesh screen.

Crystalline phases of the aquifer material were identified by matching the diffraction patterns gathered by XRD to a library of known diffraction patterns. A semi-quantitative analysis was obtained by comparing the areas of the primary diffraction peak of each crystalline phase found. This gave the relative concentrations of the different phases. Changes in the relative abundance of iron phases in the aquifer may be represented by the changes in the ratio of the areas of the primary peak of that iron phase and the primary quartz peak resulting from the ubiquitous quartz. A decrease over time in this ratio for samples from a particular site location represent a probable decrease in the concentration of that iron phase over time. For one nearest field location at mid-depth (i.e. the region of the aquifer that had the greatest dissolved aqueous indicators of microbial activity), detailed narrow scans over a main Fe oxide peak ($2\theta \approx 44.4$) also were collected in order to observe any changes in the Fe oxide content of the sediments. This peak position is not the primary diffraction peak for goethite, but more intense goethite peaks are obscured by quartz peaks that dominate the diffraction pattern.

The total abundance of all iron phases was determined by acid extraction for core samples; these extractions were performed on samples from each sampling period in order to monitor changes in the iron mineralogy over time.

The January 1996 aquifer samples were used as a baseline for iron-phase extraction analysis. Since the hydrocarbon plume had not yet reached the near field sampling locations by January 1996, variability in their extract concentrations is representative of the heterogeneity of the aquifer material. The variability of the iron-phase concentrations in January 1996 samples was as high as variations observed at the same location over time (within the limitation that same sample location can not be cored repeatedly). Thus, high local heterogeneity caused the iron phase extraction methods to be useful primarily for documenting the presence or absence of the iron-phases, and not necessarily for assessing changes or trends in iron phase abundance.

Important morphological and chemical data were obtained by SEM/EDS. Randomly chosen particles from each core sample were analyzed for their elemental concentrations. Iron to silicon ratios were averaged for all the particles tagged for a given sample. Comparison of these ratios among samples collected from the same area in the aquifer but at different times allows for the estimation of changes in the abundance of iron phases during the NATS experiment.

Changes in the SSA of aquifer sediments collected from the same area also were assessed. The direction of change (increase or decrease) depends on the forms of the Fe(III) phases consumed and of any secondary phases formed as the result of microbial activity. However, the existence of a change in SSA with time may be an indicator of microbial use of Fe(III) phases. For example, if Fe(III) formed high surface area coatings over the majority of the sediment particle surface, loss of the Fe(III) oxide coatings may result in a decrease in the sediment SSA as quartz, with a much lower overall SSA, is exposed. Alternatively, if the Fe(III) phases are more three-dimensional ("chunkier") and located primarily in cracks and other high SSA surface defects of the underlying quartz particles, consumption of Fe(III) may result in an increase in SSA of the sediment. Additionally, secondary phases with distinct but unknown (likely high) SSA may form. In summary, we may see a change in SSA upon loss of incipient Fe(III) phases but cannot predict the magnitude or direction of that change *a priori* without additional information on the form of the Fe(III) phases present. SEM/EDS and XRD data may be used to estimate the forms of iron phases and thus be used to strengthen the interpretation of changes in SSA.

Laboratory Experiment Solids

The solids from the laboratory experiments (both the microbial growth and the magnetite experiments) were also subjected to the same suite of analytical methods as for the field solid samples. Additionally, the supernatants were analyzed for Fe(II) and acetate. The use of these data for each experiment is described below.

For the microbial growth experiment, sediments were characterized only after the six months reaction time elapsed; replicate analyses were averaged for each method. Results for the vials with added microbes and sediments were compared with various controls: sediments only with no added microbes;

microbes only; media with no sediments or microbes; abiotic control containing sediments but no microbes. Since all the experiments were conducted on a homogenized mixture of sediment, the variability in results, outside the inherent variability determined for the analyses, should have been due to the different treatments. Relative amounts of iron reduced in the different treatments, as well as the total available iron in the sediments, were determined by iron extraction of the sediments. Changes in the iron mineralogy were assessed by the changes in the relative ratios of peak areas from XRD analyses and from the silica and iron ratios from SEM/EDS analyses. Changes in surface area after reaction were also determined and evaluated in light of the known changes in iron mineralogy as determined from iron extractions, XRD and EDS/SEM. Additionally, the supernatant from the microbial growth laboratory experiments was monitored for acetate and toluene reduction and Fe (II) production. Differences in Fe(II) and acetate among treatments provides insight into the microbial activity of the experimental vial.

For the magnetite experiment, similar solids and solution analyses were performed. As for the microbial experiments, the aqueous Fe(II) and acetate analyses were used to document microbial activity; the Fe(II) analyses (specifically loss of Fe(II) over time) also provided insight into the resorption of Fe(II) by the solids in the systems. The Fe(II) content of the solids was also directly determined to assess Fe(II) sorption. The XRD data provided insight into the type of secondary phases formed under abiotic and biotic oxidation of organics. Comparison of results for the abiotic and biotic vials allowed for identification of secondary phases that are (or are not) indicative of biodegradation of organics in these systems. EDS/SEM allowed for identification of morphological differences in the secondary iron phases, in particular of the magnetite.

4. Field experiment

This study was designed to investigate changes in the iron-bearing phases in the Columbus AFB aquifer. As noted above, the NATS site has low concentrations of other oxidants, so microbially-mediated iron(III) reduction of the emplaced hydrocarbons will be the primary oxidant once the oxygen has been depleted. This field experiment was designed, in part, to characterize changes in the Fe (III)-bearing phases due to microbial degradation and to assess the availability of these phases to support hydrocarbon degradation.

4.1. NATS Site Core Sampling methods and core locations

Four transects were sampled by sediment coring several times during each year of the study. For each core, a truck mounted auger drill was used to remove material to the depth of the top of the desired core, and a split barrel core tube with sterile plastic liner was driven to appropriate depth with a hydraulic hammer. The four sediment core transects are defined as: above-field (A1-A3), 10 meters up-gradient from the source; near-field (N1-N5), 2 to 10 meters down-gradient from the source; and mid-field (M1-M3) and far-field (F1-F3) 20 meters and 50 meters respectively down-gradient from the source (Figure 4). Three to five core samples were taken in each area and sub-sampled at three depths below the water table, approximately 4, 6 and 8 meters below the surface, denoted as "top," "middle," and "bottom," respectively. The two very near field locations (N4 and N5) were added after the start of the experiment because of observed nearest-field changes in the aqueous chemistry; therefore, there are no baseline data for these locations (i.e. no Jan 1996 data). Cores with the same sample designation but taken at different times were near one another, generally within 1m, but could not be co-located due to the unavoidable disruption of the aquifer by coring.

Each core sediment sample was immediately placed in a glass vial and frozen to limit oxidation of Fe (II) in the samples. The samples were then shipped (TO WHERE) on ice by overnight mail. Upon receipt in our laboratory, the samples were stored at -80°C until they could be processed. Processing of aquifer samples consisted of freeze-drying and then sieving to remove all particles larger than 0.45 mm; processed samples were then stored at room temperature. This <0.45 mm fraction was used for all characterization procedures (described above in the methods section).

The aquifer sediments were sampled five times during the experiment: January, April and September 1996, and March and September 1997, and was sampled two more times in 1998 and in 1999. Sampling dates were determined, in part, by groundwater concentration data collected by other NATS investigators. These data included pH, dissolved oxygen, total organic carbon, aqueous Fe (II) and methane concentrations, and $^{12}\text{C}/^{13}\text{C}$ isotope ratios in groundwater near the coring sites (see Appendix for selected data). Our collaborators used these concentration data to support their investigations regarding hydrocarbon transport and degradation by microbial processes.

The January 1996 samples provided the background data to which all other samples were compared¹. After this time, a distinct, migrating hydrocarbon plume was observed with the highest concentrations about the middle depth. Sediment samples analyzed from this depth were deemed the most likely to

¹ This is justified because the hydrocarbon plume had not migrated significantly down gradient in the 40 day period from the emplacement to the sampling date in January 1996.

show changes in the iron-bearing phases because of their enhanced contact with the hydrocarbon plume; most of the subsequent samples were analyzed at this depth. Select samples from other areas of the aquifer were used as a baseline in conjunction with the January 1996 samples. The samples analyzed are outlined in Table 3.

4.2. Analytical Methods for field sediments

The sediment samples collected in the field, were subjected to several analytical methods to determine the iron mineralogy. Selective iron extraction, Fe (II) analyses, x-ray diffraction, and SEM/EDS analyses, were performed on the samples listed in Table 3. These methods are listed and explained in Section 7.1.

Table 3: Samples for characterization analysis

Samples were taken from 4 meters (T = top level), 6 meters (M = middle level), and 8 meters (B = bottom layer) below grade. Full suite of analyses were performed on samples denoted as S, or the samples were taken and kept frozen for future analyses if necessary (T). Locations are denoted as A for above source, N for near source, M for mid-field, and F for far field.

Sample Name	Jan. '96	Sept. '96	March '97	Sept. '97
	T M B	T M B	T M B	T M B
A1	T T T	S T S	S T S	S T S
A2	T T T	S T S	S T S	S T S
A3	T T T	S S S	S S S	S S S
N1	T T T	S T S	S T S	S T S
N2	T T T	S T S	S T S	S T S
N3	T T T	S S S	S S S	S S S
N4		T T T	T T T	T T T
N5		T T T	T T T	T T T
M1	T T T	S T S	S T S	S T S
M2	T T T	S T S	S T S	S T S
M3	T T T	S S S	S S S	S S S
F1	T T T	S S S	S S S	S S S
F2	T T T	S T S	S T S	S T S
F3	T T T	S S S	S S S	S S S
Source Trench			T T T	T T T

4.3. Results

The results from the analyses on the field samples are somewhat difficult to characterize, due to the heterogeneity of the aquifer. There does not appear to be a marked decrease in the iron of the aquifer in general. However, some of the samples in the near field do seem to show a decrease in amorphous iron overtime, as discussed below.

XRD data provided a qualitative analysis of the crystalline phases of the aquifer. XRD data indicate that all bulk samples and fine-fractions are predominantly quartz. Minor clays (illite and kaolinite) and iron oxyhydroxide (goethite) were detected in many of the bulk samples. In addition, an exchangeable clay (smectite) and amorphous hydrated ferric oxide (ferrihydrite) were detected in the fine fractions, indicating that there are trace to minor amounts of these reactive phases in the aquifer materials. More detailed analysis, especially quantification, of the iron oxide phases by XRD is precluded because the prevalent small crystallites of iron oxides (i.e. cryptocrystalline goethite) result in broad diffraction peaks; such that phase may be identified but not readily quantified with XRD. This is exasperated for amorphous materials which produce low amplitude broad peaks over several degrees and usually cannot be easily identified in the bulk sample XRD data. Also the iron phases often were a small percentage of the total sediment, so their identification and quantification was at times difficult.

Changes in iron mineralogy were also examined by more detailed XRD analysis (smaller steps and much longer counting times) of the principal iron oxide peak separated from quartz peaks, for aquifer material from the location and depth where aqueous chemistry data showed greatest evidence of microbial activity (borehole MW-10, mid-level). First, the expected changes in this region of the XRD pattern were discerned by comparing CDB-extracted and as-received sediment, which clearly shows that the broad peak at $\approx 44.4^\circ 2\theta$ is due to extractable iron. (Figure 5). Similarly, there is an apparent decrease in iron content over time at the location of the MW-10 samples. The broad peak at $\approx 44.4^\circ 2\theta$ has largely disappeared in the March 1997 sample relative to earlier sampling dates, implying significant removal of the available iron. Furthermore, these results demonstrate that the incipient microbial community can use the more crystalline iron oxides and are not limited to amorphous iron oxide.

Total iron concentrations determined from 5N HCl extractions show the heterogeneous nature of the aquifer. In general, the field samples do not show any consistent decrease in iron concentration except in the near field at N-2 (middle level), N-4 (top and middle levels) and N-5 (bottom level) (Figure 6, Appendix 12.2 Table 4).

The EDS data, listed in Appendix 12.2 Table 2, also shows a distinct decrease in surface iron concentrations in the near field. The Fe/Si ratio always is lower in the final (Sept 1997) samples than in the preceding sample, and is at its lowest value in six of these eight samples. One of these sampling locations was not analyzed for the middle sampling times (Sept 1996 and March 1997). However, of the remaining seven samples, the Fe/Si ratio *increased* for these middle sampling times, with the Sept. 1997 final samples having a two to five fold decrease in Fe/Si compared with the March 1997 samples. This increase may reflect mobilization of Fe(II) from material even closer to the source zone by to microbial reduction, with subsequent reprecipitation in the near field. Thus, "upgradient" microbial activity may result in an increase in hydrocarbon oxidizers (i.e. ferric iron phases) in the downfield. Such precipitates are likely to be less crystalline than the pre-existing iron oxides and thus may be more available for microbial activity. The EDS data also illustrate the heterogeneity of the aquifer, as the initial Fe/Si ratio, i.e. that for all samples taken in January 1996, ranged from 0.88 to 1.65 with an average of 1.13.

Amorphous iron concentrations of the sediment as determined from the 1N HCl extraction are listed in Table 6. In most areas of the aquifer, the data show that the sediments are heterogeneous. Additionally, the values for the last sampling time, Sept 1997, are anomalously low for most samples including in the far field; this indicates that for some reason, the extraction of amorphous Fe from these samples may have been incomplete. As such, it is difficult to ascertain temporal changes in the amorphous iron content of the sediments. Nonetheless, it is clear that there is an *increase* in amorphous iron at the mid-depths of the nearest field samples (N-4 and N-5) between Sept 1996 and March 1997. These data also suggest mobilization of Fe(II) from within and/or nearer the source and subsequent precipitation of new iron phases. Note that other sample locations also have increases in total amorphous Fe, including above source locations, and thus these data are not conclusive. However, the N-5 middle level increase in total amorphous Fe is accompanied by a *decrease* in amorphous Fe(II) (Table 6) which supports the argument that this increase in total amorphous Fe is at least in part due to formation of new iron phases; all other increases in total amorphous Fe have a corresponding increase in amorphous Fe(II) (not including the suspect Sept 1997 amorphous Fe data).

The amorphous Fe(II) content of the sediments, listed in Table 7, also shows that the samples from the field are heterogeneous although in general they are very low in Fe(II). Sediment samples from section N-5 have Fe(II) concentrations that are approximately two times higher than seen in the rest of the aquifer samples. Ferrous iron released from microbial activity is not conservative, i.e. it may be removed from the groundwater via sorption processes. Thus increases in Fe(II) may be an indicator of Fe(II) release from upgradient microbial degradation processes and subsequent resorption onto sediment phases. However, this would only occur appreciably near the site of Fe(II) release, that is, before transport of Fe(II) in groundwater to oxygenated regions where it is precipitated as Fe(III) oxides.

Table 6: Amorphous Iron in Field Samples

Date and location	Top % Fe (w/w)	Middle % Fe (w/w)	Bottom % Fe (w/w)	Date and Location	Top % Fe (w/w)	Middle % Fe (w/w)	Bottom % Fe (w/w)
1/96 A-1	0.10	0.12	0.16	1/96 M-1	0.10	0.09	0.06
9/96 A-1		0.36		9/96 M-1		0.17	0.04
3/97 A-1		0.18		3/97 M-1		0.05	
9/97 A-1		0.01		9/97 M-1		0.01	
1/96 A-2	0.11	0.11	0.08	1/96 M-2	0.08	0.08	0.06
9/96 A-2		0.16		9/96 M-2		0.05	
3/97 A-2		0.09		3/97 M-2		0.13	
9/97 A-2		0.01		9/97 M-2		0.01	
1/96 A-3	0.22	0.07	0.04	1/96 M-3	0.20	0.06	0.22
9/96 A-3				9/96 M-3			
3/97 A-3				3/97 M-3		0.03	
9/97 A-3		0.01		9/97 M-3			
1/96 N-1	0.13	0.11	0.05	1/96 F-1	0.14	0.24	0.03
9/96 N-1				9/96 F-1		0.05	
3/97 N-1		0.03		3/97 F-1		0.02	
9/97 N-1				9/97 F-1			
1/96 N-2	0.11	0.11	0.38	1/96 F-2	0.16	0.03	0.10
9/96 N-2		0.06		9/96 F-2		0.11	
3/97 N-2		0.04		3/97 F-2		0.05	
9/97 N-2		0.01		9/97 F-2		0.00	
1/96 N-3	0.12	0.08	0.19	1/96 F-3	0.02	0.04	0.09
9/96 N-3		0.05		9/96 F-3			
3/97 N-3		0.03		3/97 F-3			
9/97 N-3		0.00		9/97 F-3			
9/96 N-4	0.09	0.07	0.11				
3/97 N-4	0.07	0.13	0.02				
9/97 N-4	0.01	0.00	0.02				
9/96 N-5	0.06	0.16	0.24				
3/97 N-5	0.03	0.37	0.04				
9/97 N-5	0.01	0.01	0.01				

Table 7. Fe (II) in field samples

Date and Location	Top % Fe(II) (w/w)	Middle % Fe(II) (w/w)	Bottom % Fe(II) (w/w)	Date and Location	Top % Fe(II) (w/w)	Middle % Fe(II) (w/w)	Bottom % Fe(II) (w/w)
1/96 A-1	0.009	0.008	0.006	1/96 M-1	0.005	0.008	0.010
9/96 A-1		0.013		9/96 M-1		0.012	
3/97 A-1		0.013		3/97 M-1		0.008	
9/97 A-1		0.009		9/97 M-1		0.012	
1/96 A-2	0.007	0.011	0.009	1/96 M-2	0.009	0.007	0.003
9/96 A-2		0.014		9/96 M-2		0.011	
3/97 A-2		0.007		3/97 M-2		0.011	
9/97 A-2		0.005		9/97 M-2		0.008	
1/96 A-3	0.015	0.007	0.006	1/96 M-3	0.006	0.004	0.021
9/96 A-3				9/96 M-3			
3/97 A-3				3/97 M-3		0.003	
9/97 A-3		0.004		9/97 M-3			
1/96 N-1	0.007	0.009	0.002	1/96 F-1	0.009	0.019	0.002
9/96 N-1				9/96 F-1		0.003	
3/97 N-1		0.001		3/97 F-1		0.001	
9/97 N-1				9/97 F-1			
1/96 N-2	0.004	0.010	0.012	1/96 F-2	0.019	0.002	0.012
9/96 N-2		0.007		9/96 F-2		0.007	
3/97 N-2		0.014		3/97 F-2		0.003	
9/97 N-2		0.004		9/97 F-2		0.002	
1/96 N-3	0.008	0.015	0.013	1/96 F-3	0.001	0.008	0.012
9/96 N-3		0.007		9/96 F-3			
3/97 N-3		0.004		3/97 F-3			
9/97 N-3		0.003		9/97 F-3			
9/96 N-4	0.006	0.010	0.010				
3/97 N-4	0.008	0.013	0.001				
9/97 N-4	0.009	0.003	0.012				
9/96 N-5	0.010	0.028	0.010				
3/97 N-5	0.001	0.021	0.008				
9/97 N-5	0.007	0.015	0.008				

The average of the total iron in the source material listed in Table 8, also shows the heterogeneous nature of the source material. However, with consideration of the standard deviation in these data, it appears that there is a slight decrease in total iron over time.

Table 8: Average of Total Iron in the Source Material over Time

Date	Average ($\mu\text{g Fe/g}$ sediment)	Standard Deviation
Jan-96	439.63	152
Sep-96	441.42	104
Mar-97	593.77	254
Sep-97	498.46	76
Mar-98	493.13	190
Apr-99	396.12	27

4.4. Discussion

Analyses from the field study clearly show that the aquifer is heterogeneous in Iron content. This heterogeneity was expected, since similar spatial variabilities over the site region for organic carbon content and hydrocarbon sorption coefficients had been reported by MacIntyre et al. 1998. The only consistent decreasing trends in total, amorphous and Fe (II) in site aquifer material are observed in the near field, primarily in samples closest to the source emplacement (N-4 and N-5). This was expected from the solution chemistry, as these were the areas (0-10 meters from the source) that had the highest dissolved hydrocarbon concentrations through time (Table 2). The high dissolved inorganic carbon and methane also suggests that this was an area where anoxic degradation was taking place. In addition, the samples from section N-5 after initial emplacement of the source material are approximately two to three times higher in iron (II) than the rest of the aquifer (there are no background values for this location). This section corresponds to the highest outflow from the source material and suggests that the majority of the iron reduction may be occurring in the source material itself with the Fe (II) then depositing on the nearby sediments. This increase in sediment-bound Fe(II) also demonstrates that Fe(II) upgradient may sorb to the sediments. As such, the dissolved Fe(II) concentrations can only serve as a conservative measure of microbial degradation and reduction of the amount of iron(III) oxide reduced in the affected aquifer material. Additionally, any sorbed Fe(II) may reduce the availability and reactivity of underlying Fe(III) oxides. Therefore, the remaining capacity of an aquifer to degrade hydrocarbons through dissimilatory reduction of Fe(III) oxides cannot be assessed by simply knowing the initial Fe(III) content and estimating loss of these phases via measuring aqueous Fe(II).

5. Laboratory experiments

5.1. Microbial Growth Experiments

5.1.1. Experimental Design and Sampling Methods

The laboratory experiments were designed to provide optimal conditions for microbial oxidation of organics so that corresponding changes in the iron phases of the aquifer material could be clearly identified. Three treatments were conducted. The first treatment was designed to assess if a known iron-reducing microbe (*Geobacter metallireducens*, also referred to as GS-15) is capable of reducing the iron-bearing phases present in the CAFB aquifer. The second and third examined reduction of iron-bearing phases by naturally-occurring bacteria, while using two different carbon sources, acetate and toluene (Table 9).

Table 9: Conducted Treatments

Treatment	Substrate	Microbes Used	Growth Medium
I	CAFB aquifer material	<i>Geobacter metallireducens</i>	Acetate
II	CAFB aquifer material	Natural consortium from CAFB	Acetate
III	CAFB aquifer material	Natural consortium from CAFB	Toluene

The sediment used in each of the treatments consisted of a freeze-dried and homogenized mixture of sediments taken above gradient from the field emplacement at the NATS site. Thus all treatments used a common site sediment of known mineralogy and surface area that allows for comparisons among the treatments. Freeze-drying the sediment was thought to severely reduce the activity of the naturally occurring bacteria without significantly changing the mineralogy of the aquifer material. Autoclaved (120°C, 20 PSI, for 60 minutes) sediment was analyzed by XRD to determine if the mineralogy changed. As no noticeable change occurred, autoclaving was used to sterilize the sediment in each treatment and control. A NATS collaborator, Derek Lovely (University of Massachusetts, Amherst) supplied the *Geobacter metallireducens* culture. The natural consortium of bacteria used here was gathered in September 1997. Sediment and pore water from an area in the aquifer with manifest ongoing microbial activity established by other NATS investigators was transferred by syringe into sealed, autoclaved serum vials containing a sterile acetate or toluene medium provided with an appropriate vitamin and mineral mixture. This encouraged growth of at least some of the natural consortium of bacteria. Table 10 shows the medium used for culturing GS-15 (Lovley, 1988)

and the naturally occurring bacteria. The GS-15 was initially cultured in this medium with the addition of an iron oxy-hydroxide gel, of which a small amount transferred with the inoculate.

Table 10: Acetate medium for GS-15 and naturally occurring bacteria

Constituents	g/L deionized water
NaHCO ₃	2.5
KCl	0.1
NH ₄ Cl	0.5
NaH ₂ PO ₄ H ₂ O	0.6
NaCH ₃ COO	2.7

Treatment I used the acetate medium and the the Fe (III)-reducing microbe *G. metallireducens*. A preliminary version of this experiment, conducted by our collaborators, has previously shown some success in altering the iron-bearing phases. Treatment II used the naturally occurring consortium of bacteria as the Fe(III) reducer and the acetate medium as the electron donor. Treatment III also used the naturally occurring consortium of bacteria, but instead used a toluene medium; this medium was the same as the acetate medium except for the substitution of approximately 280 mg toluene per liter water for the acetate. Toluene is a component of the mixture of hydrocarbons emplaced in the field experiment. *G. metallireducens* has been successfully grown on toluene and it was hoped that the naturally occurring bacteria would also be able to use toluene as an electron-donor. Fe (II) production and, where appropriate, acetate reduction, were analyzed as indicators of bacterial growth.

For all three treatments, the sediment mixture, appropriate food source/water mixtures and a vitamin/mineral supplement were combined in 100 ml serum vials fitted with a rubber septum, keeping a head-space of 80% N₂/20% CO₂. The mixtures were then autoclaved at 120 °C and 20 PSI for 60 minutes to sterilize the sediment, inoculated with the appropriate microbes, and incubated at 30°C. All treatments were conducted in triplicate.

Three different controls were studied. Abiotic controls containing the sediment mixture, the appropriate medium and vitamin/mineral mixtures were used to assess the extent of iron reduction in the absence of microbial activity. Controls without sediments, but with the medium, vitamin/mineral mixtures and inoculate were used to determine the amount of iron transferred with the inoculate. Finally, controls with only the media and vitamin/mineral mixture were analyzed to establish baseline concentrations of the organics and Fe. The experimental treatments were allowed to incubate at 30°C for six months. After the incubation period, the samples were opened and filtered to separate the

solids, for characterization by a suite of methods, and the supernatant, for Fe (II) and acetate analysis. The filtered solids were rinsed twice with small aliquots of DI water and freeze dried for iron extraction.

After the experiment, there was concern that the sediments were not completely sterilized and that naturally occurring microbes grew in all sediment containing vials. Specifically, the abiotic controls had considerable generation of Fe (Tables 11, 12). Therefore, an additional set of vials, labeled sediment control, were prepared that included the sediment mixture, growth media with the vitamin and mineral mixture. These vials were autoclaved like the three above treatments, but were allowed to incubate at 30°C for only three days; this three-day abiotic control is referred to as the “control” whereas the 6-month “abiotic” treatment is now referred to as “sediments only” since it appears as if it was not an abiotic system.

5.1.2. Results

Total iron in solution (Table 11) was similar in all three treatments for all vials containing sediments, ranging from 10.6-12.9 ppm. In the vials with only naturally occurring bacteria and no sediments, the total iron concentration was ≈ 1 ppm iron. However, in the vials with the GS-15 bacteria, the total iron concentration was considerably higher (8 ppm total iron). All vials with only media and the three-day abiotic control (“Seds. Only”) had iron concentrations below the detection limit of 0.05 ppm.

Table 11: Amount of Total Fe (mg/L) in solution

Naturally Occurring

<u>Acetate Vials</u>	Triplicate 1	Triplicate 2	Triplicate 3	Average Fe (mg/L)
Seds. Only	10.00	14.25	10.44	11.57
Seds.+ Bugs	12.45	10.62	10.90	11.32
Bugs Only	1.04	1.10	0.87	1.00
Media Only	<0.05	<0.05		<0.05

Toluene Vials

Seds. Only	11.22	10.91	14.45	12.19
Seds.+ Bugs	11.36	9.52	10.75	10.55
Bugs Only	1.02	0.95	0.98	0.98
Media Only	<0.05	<0.05		<0.05

GS-15 Innoculates

Acetate Vials

Seds. Only	14.16	4.88	18.74	12.59
Seds.+ Bugs	14.38	12.04	12.30	12.90
Bugs Only	8.66	7.32	8.30	8.09
Media Only	<0.05	<0.05		<0.05

Abiotic Control

Seds. Only	<0.05	<0.05		<0.05
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The amount of reduced iron in solution is listed in Table 12. In the vials with sediments only, the Fe (II) concentration ranged from 3.5 to 4.2 ppm. In the sediment-containing vials inoculated with the naturally occurring bacteria, the toluene and acetate vials had concentrations of 4.2 and 3.8 ppm, respectively. The sediment-containing vials inoculated with GS-15 had lower average Fe (II) concentrations (2.9 ppm). These Fe(II) concentrations constitute $\approx 34\%$, 40% , and 23% , respectively of the total Fe for each system, natural-consortium acetate medium, natural consortium toluene medium, and GS-15 acetate medium. The media vials inoculated with the GS-15 had Fe (II) concentrations of 2.4 ppm, whereas the media vials with the naturally-occurring bacteria inoculate had Fe (II) concentrations of approximately 0.4 ppm. Although the total Fe(II) is higher for the GS-15 bugs only samples, the percent Fe(II) of the total is lower (about 30%, vs. 40% for the natural consortium bugs-only samples, both media). The vials containing just the media in all three treatments had concentrations approximately 0.04 ppm Fe (II), which suggests that most of the Fe is present in its reduced form in these treatments. The sediment control vials that were incubated for only 3 days had no detectable Fe (II) in solution.

Table 12: Concentration of Fe (II) (ppm) in Solution

Naturally Occurring

<u>Acetate Vials</u>	Triplicate 1	Triplicate 2	Triplicate 3	Average Fe (ppm)
Seds. Only	3.49	4.70	3.56	3.92
Seds.+ Bugs	4.07	3.68	3.65	3.80
Bugs Only	0.44	0.32	0.39	0.38
Media Only	0.03	0.04		0.04

Toluene Vials

Seds. Only	4.15	3.62	4.70	4.16
Seds.+ Bugs	3.77	3.16	5.70	4.21
Bugs Only	0.32	0.42	0.44	0.40
Media Only	0.04	0.04		0.04

GS-15 Innoculates

Acetate Vials

Seds. Only	4.72	2.08	3.83	3.54
Seds.+ Bugs	4.65	3.67	0.47	2.93
Bugs Only	2.62	2.12	2.44	2.39
Media Only	0.03	0.03		0.03

Abiotic Control

Seds. Only	<0.1	<0.1		<0.1
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The average acetate consumed for the three replicates was determined for both the natural consortium and GS-15 treatments (Table 13). Note that a similar determination of toluene consumed by the natural consortium in Treatment II cannot be made because of analytical difficulties (see Methods in Chapter 7). Acetate consumed is calculated by subtracting the acetate

concentration in the reacted vials from the acetate concentration in the media, and multiplying by the volume of media in each vial. For treatment III (GS-15 in acetate), acetate consumption was greatest in the presence of both sediments and microbes, lower in the vials with sediments only, and lowest in the "bugs only" and "media only" vials. In contrast for treatment I (natural consortium in acetate) more acetate was consumed in the sediment only vials than in the sediment and bacteria vials, with the lowest consumption again for the "bugs only" and "media only" vials. Additionally, acetate consumption was higher in the vials with the naturally-occurring bacteria compared with the GS-15.

Table 13: Average Acetate Consumed (μg)

<u>Naturally Occurring</u>	<u>Average</u>
<u>Acetate Vials</u>	<u>Acetate</u>
	<u>Consumed</u>
	<u>(μg)</u>
Seds. Only	12920
Seds.+ Bugs	10005
Bugs Only	8751
Media Only	
 <u>GS-15 Inoculates</u>	
<u>Acetate Vials</u>	
Seds. Only	3619
Seds.+ Bugs	8978
Bugs Only	1743
Media Only	
Abiotic	
Control	
Seds only	

The amount of total iron from the 5N HCl extraction and the amorphous iron determined by the 1N HCl extraction for the reacted sediments are listed in Table 14. There was negligible difference between the three treatments as well as in the abiotic control. In each treatment, the total iron was about 3 ppm and the amorphous iron was approximately 1 ppm. These results indicate that the majority of the sediment iron phases was not utilized by the bacteria over the 6 month incubation period, despite the high aqueous concentrations of iron.

Table 14: Amount of Fe Extracted from Reacted Sediments

Naturally Occurring	1N Fe Extraction	5N Fe Extraction
Acetate Vials	(mg Fe/g sediment)	(mg Fe/g sediment)
Seds. Only	0.7	3.2
Seds.+ Bugs	0.9	3.4
Toluene Vials		
Seds. Only	1.1	3.5
Seds.+ Bugs	1.2	3.3
GS-15 Innoculates		
Acetate Vials		
Seds. Only	1.0	3.2
Seds.+ Bugs	0.8	3.1
Abiotic Control		
Seds. Only	0.6	3.1

The surface iron concentrations of the reacted sediments as determined by EDS are listed in Table 15. In each of the three treatments, surface iron concentrations (weight % Fe) normalized to silica concentrations increased with reaction of the sediments with the bacteria, suggesting readsorption of solubilized iron. However, as the aqueous iron concentrations are similar with or without added microbes, these results may also indicate a difference in the form of resorbed Fe whereby resorbed iron covers the surface in a manner that more effectively masks the underlying mineral surfaces in the presence of bacteria.

Table 15: Surface Fe (wt %, normalized to Si wt%) Concentration of the Sediments Determined by EDS

<u>Naturally Occurring</u>	
<u>Acetate Vials</u>	Average wt.% Fe
Seds. Only	0.78
Seds.+ Bugs	0.88
<u>Toluene Vials</u>	
Seds. Only	0.60
Seds.+ Bugs	0.66
<u>GS-15 Innoculates</u>	
<u>Acetate Vials</u>	
Seds. Only	0.68
Seds.+ Bugs	0.74
<u>Abiotic Control</u>	
Seds. Only	0.87

N₂-BET specific surface areas (SSA) are listed in Table 16. In both treatments involving the naturally occurring bacteria, surface area increased with reaction of the sediments with the bacteria. Additionally, the SSA of the control is similar to the sediment-only vials of these two treatments. In the treatment inoculated with the GS-15, SSA was higher than the naturally occurring treatments and *decreased* with reaction of the sediments with the bacteria.

Table 16: Average Specific Surface Area (m²/g) of Reacted Sediments

<u>Naturally Occurring</u>	Average Surface Area (m ² /g)
<u>Acetate Vials</u>	
Seds. Only	1.3
Seds.+ Bugs	2.5
<u>Toluene Vials</u>	
Seds. Only	1.9
Seds.+ Bugs	2.4
<u>GS-15 Inoculates</u>	
<u>Acetate Vials</u>	
Seds. Only	3.7
Seds.+ Bugs	2.8
Sediment Control	1.5

5.1.3. Discussion

It is apparent for several reasons that autoclaving the sediments at 120°C and 20 PSI was insufficient to kill off all the naturally occurring bacteria. The concentration of total iron and iron (II) in solution is very similar for the vials with sediments only and the vials with sediments and bacteria in all three treatments. And when compared to the iron concentrations in the three-say abiotic control, which is below detection limit, something has definitely reacted with the sediments over the course of the 60 day treatments. Also, the acetate consumption in the sediment only vials was quite high, for the naturally occurring bacteria treatment, higher than in the vials inoculated with the bacteria. So, over the six-month incubation period, the naturally occurring bacteria in the sediments that survived the autoclaving had plenty of time to establish a viable population.

The analysis of surface iron concentration normalized to silica concentrations does show a slight increase in iron coating the particles. This suggests that solubilized iron has been redeposited on the surface of the particles, perhaps in a form different than for the original iron phases.

The amount of the extractable iron in the sediments verses the sediments and bacteria did not differ significantly between treatments or from the abiotic control. So, it appears that six months is not enough time to show a significant reduction in the iron phases coating the sediments in a laboratory setting. However, this may in part be an artifact of the experimental design. In an aquifer system, any release iron is continually removed via groundwater transport whereas high levels of aqueous iron are achieved in a batch laboratory experiment. These high iron concentrations may suppress further microbial activity and also allow for readsorption of Fe which may "poison" the iron oxide surfaces for further microbial degradation.

The larger surface areas seen in the GS-15 treatment in comparison to the two naturally occurring treatments could be due to the transfer of the iron oxy-hydroxide gel on which the GS-15 was cultured. Such material has a very high specific surface area, $600 \text{ m}^2/\text{g}$ for fresh precipitate (Dzombak and Morel, 1990), and so, even a small amount could affect the surface area measured. Furthermore, this amorphous iron is often considered more available for microbial reduction than the relatively more crystalline iron phases present in the sediments. As such, the amorphous iron may be preferentially consumed, thereby reducing the surface area of the system. This could explain the decrease seen in the surface area seen between the sediment only and sediment with GS-15 vials, where the surface area increased between the same vials that were inoculated with the naturally occurring bacteria that were cultured on the iron oxy-hydroxide gel. Unfortunately, because there was not a clear decrease between all three treatments, surface area analyses were not conducted extensively in the field experiment.

In summary, there was considerable consumption of Fe(III)oxides in the CAFB sediment by both GS-15 and naturally-occurring bacteria. The high levels of iron released may have been partially resorbed. The effects of both the high Fe concentrations and this resorption are unknown, but may have caused a decrease in microbially-assisted dissolution of the iron oxides. The majority of the sediment iron oxides were not consumed during the six month incubation periods. These results indicate that either the capacity for iron reduction has not been reached (and thus longer incubation times are needed) or that the initial capacity diminishes over time due to the build up of iron in the system. While such a build up of aqueous iron is not expected in the field because of groundwater movement, a similar decrease in capacity may occur in the field as iron released due to microbial activity is transported down-field and subsequently resorbed onto the aquifer materials.

5.2. Magnetite Experiment

Magnetite has been observed in contaminated aquifers under iron reducing conditions and is considered to be a by-product of microbial iron reduction. Iron-reducing bacteria use ferric iron as a terminal electron acceptor, with an overall reaction of reducing solid Fe (III) oxides to aqueous Fe (II).

Magnetite is considered to be formed by a matrix rearrangement and water expulsion when aqueous Fe (II) sorbs into amorphous or poorly crystalline Fe (III) oxides on the surface of the aquifer material. If magnetite is formed only in the presence of iron-reducing microbes, then magnetite production may be an indicator of microbial degradation of hydrocarbons. This study was designed to test for abiotic production of magnetite under similar culture conditions required for microbial production of magnetite.

5.2.1. Design and Sampling Method

Batch cultures of *Geobacter metallireducens* (GS-15) were grown under strict anaerobic condition using the same growth media, hydrous iron oxide (HFO) gel, and method as outlined in section 9.1.1. Two treatments were designed, one group inoculated with GS-15 and the other left abiotic. Triplicate vials were set up to which Fe (II), in the form of an aqueous solution of FeCl_2 , was added in the concentrations of 50, 100, 200, 400 and 800 mg /L. Abiotic controls were incubated along with the microcosms containing bacteria for five months at 30°C.

After one month, 5 ml aliquots of the solution were removed by syringe filter and tested for Fe (II) and acetate concentrations. After 5 months, the vials were opened, and the samples were vacuum filtered through a 0.45µm cellulose membrane. The filtrate was sub-sampled for total iron, Fe (II) and acetate analyses following the sample methods used for the field experiment (see section 7.1).

The filtered sediment was then rinsed twice with a small amount of DDI water to minimize the amount of Fe (II) from the pore water, and freeze dried. The dried samples were then homogenized and sub-sampled for 1 N HCl extraction, XRD analysis, and SEM/EDS analysis (see section 7.1 for details).

An additional 5 – 10 g sample of each dried sample was placed on a plastic weighboat and swirled over a strong magnet to concentrate the magnetized particles. The magnetic fraction was subjected to XRD and SEM/EDS analysis as for the bulk sediment; prior to SEM/EDS, this fraction was examined under an optical microscope in order to initially identify the characteristics (size and morphology) of the magnetite. Magnetite could be distinguished from associated HFO by its optical properties (e.g. degree of opaqueness and octahedral structure).

5.2.2. Results

Average acetate concentrations in the biotic vials after 5 months is significantly less than in the abiotic vials, except in the 800 ppm Fe (II) addition. These results show that there was degradation of the acetate, most likely due to microbial activity (Table 18). Note, however, that there appears to be a loss of

acetate in all vials, most likely due to an unknown amount of hydration of the ammonium acetate (the initial concentration of acetate added as media was 2.7 mg/L NaCH_3COO , which is equivalent to 1.9 g/L CH_3COO^+). Furthermore, the acetate loss in abiotic vials was variable, with greater loss in the 0 and 800 ppm (lowest and highest Fe (II) additions). The difference between the abiotic and biotic vials is generally greater at lower Fe (II) additions, suggesting a possible effect of Fe (II) on microbial activity (Table 18).

Table 18: Average Acetate Concentrations in Vials after 5 months

Sample designations refer to the amount of Fe(II) added in mg/L, with A denoting the abiotic samples and B the vials with microbes. Const. 80 samples were handled slightly different; The controls were vials with no added Fe(II) for each of the abiotic and biotic systems.

Sample Designation	Acetate ($\mu\text{g/L}$)
50 A	1527
50 B	1205
Const. 80 A	1563
Const. 80 B	1258
100 A	1661
100 B	1447
200 A	1576
200 B	1324
400 A	1470
400 B	1253
800 A	1320
800 B	1431
CONTROL A	1440
CONTROL B	1191

Microscopic Data

The optical microscope used was not outfitted with a camera, so no pictures are available, although descriptions of the observations are reported here. Size estimates were based on graduated crosshairs on the optical microscope. In general, the magnetite particles were larger in the biotic samples than in the abiotic samples. Examination of the magnetic separates by SEM also clearly shows differences between magnetite formed in the abiotic samples versus the biotic samples. Specifically, the biotic samples contained larger magnetite crystals; typically 100-200 μm , with some particles 0.5 - 1.5 mm. Abiotic samples had magnetite crystals typically 10-50 μm with the largest particles ~ 100 - 200 μm , or near the average size seen for the biotic samples.

Table 17 shows the concentration of Fe (II) in solution after 1 month and 5 months. After 1 month, the Fe (II) concentrations in the abiotic vials are all lower than those initially introduced to the vials. This indicates that some Fe (II) in the abiotic vials has sorbed into the iron gel. Fe (II) production in biotic vials keeps the ferrous iron concentrations higher than in the comparable abiotic vials. However, Fe (II) concentrations are lower than the initial values in the vials with ≥ 200 mg/L initial Fe (II), again suggesting some reabsorption into the HFO. After 5 months, there is little Fe (II) remaining in solution in either the abiotic or biotic vials, with moderate levels only for the highest initial Fe (II) 800 mg/L vials. These results suggest that most of the Fe (II) is incorporated into the ferric oxide in both biotic and abiotic vials.

Table 17: Fe (II) Concentration (mg/L) in Vials after 1 and 5 Months

Sample Name	1 Month Fe (II) Concentration (ppm)	5 Month Fe (II) Concentration (ppm)
CONTROL A1	0.49	0.22
CONTROL A2	-0.25	0.26
CONTROL B1	119.51	16.48
CONTROL B2	128.26	16.25
50 A1	5.30	3.27
50 A2	4.73	2.53
50 A3	7.27	4.91
50 B1	247.68	
50 B2	160.63	
100 A1	21.22	6.51
100 A2	18.86	
100 A3	19.74	
100 B1	210.50	15.64
100 B2	98.08	14.29
100 B3	108.14	15.58
200 A1	40.44	8.02
200 A2	42.45	15.06
200 A3	40.35	17.17
200 B2	200.00	16.00
200 B3	158.88	15.06
400 A1	89.76	36.59
400 A2	130.01	
400 A3	112.95	
400 B1	368.85	15.73
400 B2	265.18	10.79
400 B3	296.24	16.85
400 B3	296.68	
800 A1	765.70	87.68
800 A2	560.98	
800 B1	682.59	90.24
800 B2	753.46	87.12
800 B3	783.20	96.40

XRD Data

The mineralogy of the abiotic and biotic systems was distinct after 5 months time. Magnetite formed in all biotic vials (Figure 7a), whereas XRD diffraction patterns of the abiotic vials show no evidence of magnetite formation (Figure 7b). Despite microscopic and magnetic evidence, the very small crystalline size and low abundance of magnetite may have made XRD analysis inconclusive for the presence of magnetite in the abiotic vials. Residual ferrihydrite (amorphous hydrated ferric oxide) is present in all abiotic and biotic vials; as evidenced by the large "blob-like" peak in the 40-48 2θ range. The biotic vials to which ≤ 100 ppm Fe (II) was added contain no goethite (ferric oxyhydroxide, α -FeOOH); otherwise, goethite formation increases with increasing Fe (II) addition. Goethite formed in all abiotic vials except the controls. Siderite (ferrous carbonate, FeCO_3) formed in all biotic vials, except the 800 ppm Fe (II) additions; the greatest abundance of siderite was in the 200 ppm Fe (II) additions. Notably, this reduced iron phase, siderite, was not detected in any abiotic vial. Hematite (ferric oxide, α - Fe_2O_3) formed in vials with 200 or 400 ppm Fe (II) additions for the biotic systems and all abiotic vials except the 800 ppm Fe (II) additions.

5.2.3. Discussion

As evidenced by magnetic affinity, optical microscopy and SEM/EDS, magnetite was formed in all the biotic vials and in the abiotic vials to which more than 200 ppm Fe (II) was added. After 1 month, the iron gel in each of the abiotic vials sorbed some of the Fe (II) in solution. After 5 months almost all of the excess Fe (II) introduced into the vials, as well as the Fe (II) produced by the microbes, was sorbed to the iron gel. As sorption of Fe (II) into the iron gel is the first step of magnetite formation, the loss of aqueous Fe (II) suggests the possibility of production of micro-crystalline magnetite inside the ferrihydrite matrix. The small crystallite size of this first formed magnetite is not detectable by powder XRD. In abiotic systems, magnetite did not form to a size or abundance detectable by XRD, even after 5 months. In contrast, the magnetite crystals were larger in the biotic systems, clearly detectable by XRD. Nonetheless, formation of magnetite in the abiotic microcosms demonstrates that magnetite crystallization is a chemical process occurring independent of microbial activity, and magnetite production in itself may not be a sufficient indicator of microbial degradation of hydrocarbons by ferric oxides.

More magnetite was formed in the biotic vials than in the abiotic. There is also a trend of decreasing magnetite production with increasing Fe (II) addition in the biotic systems, but increasing magnetite production with increasing Fe (II) addition in the abiotic systems. With increasing Fe (II) concentration, more Fe (II) is sorbed into the iron gel, forming a Fe (II)/Fe (III) complex that rearranges its matrix structure and expels water, forming magnetite. When the iron gel

becomes pre-magnetite, it is in a more reduced state and may be less effective for microbial oxidation of acetate. So, one might expect the effects on magnetite formation observed here.

Siderite, Fe(II)CO_3 , formed in the biotic systems, with the exception of the 800 ppm Fe (II) addition. The oxidation of acetate by the microbes reduces Fe (III) to Fe (II), increasing the $[\text{Fe (II)}]$, and releases CO_2 in the form of bicarbonate (the system is buffered such that the pH remains near pH 7). Once the solubility product of siderite, $K_{sp}=10^{-10.67}$, is exceeded, siderite precipitates. This can be used as an indicator of biological activity in closed systems containing Fe (II). There appears to be a maximum siderite formation with the 200 ppm Fe (II) addition. One would expect there to be less siderite formation in vials when there is less Fe (II) present, and that siderite formation would increase at higher Fe (II) concentrations. The decrease in siderite, and magnetite, in the 400 ppm Fe (II) additions and the absence of siderite formation in the 800 ppm Fe (II) addition again indicates that the excess Fe (II) sorbed in the ferrihydrite gel decreases microbial oxidation of acetate.

6. Summary

This portion of the NATS provides three important findings. First, that at least some of the natural consortium of bacteria is capable of degrading hydrocarbons in a reducing environment in the presence of iron oxides. This was shown by the amount of acetate that was consumed and the increase in Fe (II) in solution. Second, that there appears to be some use of the iron oxides in the field near the source emplacement. There was a decrease in the total iron oxides at the two core sites closest to the source, a dramatic increase in the Fe (II) in solution near the source and the presence of HC metabolites and degradation products in the groundwater. And third, that magnetite should not be used as a primary indicator of microbial degradation in the field. The laboratory study showed that magnetite was produced both biotic and abiotic systems.

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8. APPENDIX

8.1. Solution Chemistry from the Aquifer Over Time

Zone	Snap	Fe ⁺² (mg/L)	Benzene (ppb)	Toluene (ppb)	Ethylbenz (ppb)	Xylene (ppb)	Naphthalene (ppb)	DIC (mM)	CH ₄ (μM)	[NO ₃ -] (ppm)	[SO ₄ -2] (ppm)	Br (ppm)	[Cl-] (ppm)
-10 - 0m	-2	0.42						1.48					
-10 - 0m	-1	0.41						1.89	0.09				
-10 - 0m	0	0.01								4.56	1.56		6.48
-10 - 0m	1	0.41						1.99	0.23	1.34	2.39	0.04	4.55
-10 - 0m	2	0.17	0	0	0	0	0			1.86	2.05	0.04	3.80
-10 - 0m	3	0.28	0	0	0	0	0	1.95	0.23	5.26	2.02	0.05	3.44
-10 - 0m	4	0.75	0	0	0	0	0	2.02	0.28	1.96	3.14	0.05	4.58
-10 - 0m	5	0.34	0	0	0	0	0	1.98	0.11	1.69	3.39	0.01	3.28
-10 - 0m	6	0.45						2.98	1.20	1.79	5.04	0.03	4.04
-10 - 0m	7	0.28						2.32	0.30	2.14	6.74	0.06	2.74
-10 - 0m	8		0	0	0	0	0						
0-5m	-2	0.07						1.42					
0-5m	-1	0.05						1.70	0.41				
0-5m	1	0.09	0	4	22	11	15	1.83	1.37	4.69	2.46	0.36	7.03
0-5m	2	0.03	3	0	11	8	0			3.67	1.78	0.33	5.06
0-5m	3	0.62	50	355	473	203	240	1.98	7.99	1.85	1.07	3.46	5.10
0-5m	4	1.35	142	1428	1616	621	1837	2.56	8.85	2.45	1.49	1.37	5.76
0-5m	5	2.44	203	4320	1947	1533	1727	2.76	17.87	1.66	2.32	0.86	5.17
0-5m	6	2.62	48	60	70	83	160	3.20	28.58	1.87	2.09	0.51	4.68
0-5m	7	1.91	127	2018	1133	1020	934	3.58	50.33	1.67	2.56	0.52	3.67
0-5m	8		285	2474	2631	2315	1877						
5-10m	-2	0.15						1.58					
5-10m	-1	0.10						1.71	0.18				
5-10m	0	0.02	17	490	156	167	5			5.10	1.04	0.32	6.16
5-10m	1	0.20	0	13	29	34	23	1.92	0.29	5.81	1.78	0.43	7.67
5-10m	2	0.37	12	187	134	108	0			5.17	1.53	0.69	6.49
5-10m	3	0.65	40	373	401	325	155	2.20	1.84	1.80	2.79	1.97	4.84
5-10m	4	3.18	50	169	635	223	1182	2.52	4.95	3.51	2.03	0.82	5.60
5-10m	5	1.06	46	220	327	229	438	2.25	5.47	2.75	3.05	0.34	4.88
5-10m	6	1.18	43	85	95	104	267	2.47	8.11	2.58	2.52	0.39	5.02
5-10m	7	0.81	22	71	161	134	176	2.40	8.69	2.96	3.06	0.22	4.18
5-10m	8		72	29	478	568	395						

			[Cl-] (ppm)	Br (ppm)	[SO4-2] (ppm)	[NO3-] (ppm)	CH4 (µM)	DIC (mM)	Naphthalene (ppb)	Xylene (ppb)	Ethylbenz (ppb)	Toluene (ppb)	Benzene (ppb)	Fe ⁺² (mg/L)	Snap	Zone
10-15m	-2	0.39						1.43								
10-15m	-1	0.34					0.22	1.69								
10-15m	0	0.94	6.72	0.15	3.15	4.10			0	4	3	5	4			
10-15m	1	0.36	7.42	0.25	1.94	5.82	0.44	1.87	0	0	0	0	0			
10-15m	2	0.11	6.94	0.07	1.95	7.22			0	0	0	0	2			
10-15m	3	0.66	4.92	0.22	5.61	1.99	0.86	1.89	5	4	5	0	1			
10-15m	4	2.27	7.88	0.25	1.65	4.70	5.73	1.99	21	2	5	0	2			
10-15m	5	0.58	4.80	0.11	1.25	4.52	5.97	1.74	10	1	0	0	3			
10-15m	6	0.55	5.13	0.12	2.04	4.08	1.34	1.95	9	0	0	0	3			
10-15m	7	0.44	4.74	0.11	2.10	4.20	1.07	2.02	1	1	0	0	2			
10-15m	8								0	0	0	0	0			
15-20m	-2	0.01						1.54								
15-20m	-1	0.02					0.05	1.62								
15-20m	0	0.02	6.05	0.06	2.33	4.88			0	0	0	0	3			
15-20m	1	0.19	6.57	0.11	1.65	5.50	0.17	1.71	0	0	0	0	0			
15-20m	2	0.33	5.77	0.08	1.32	6.13			0	0	0	0	0			
15-20m	3	0.42	4.00	0.05	4.17	2.18	0.69	1.82	0	0	0	0	0			
15-20m	4	1.88	6.63	0.08	1.62	5.64	1.04	2.03	0	0	0	0	0			
15-20m	5	0.35	4.10	0.03	1.18	4.30	0.90	1.92	0	0	0	0	0			
15-20m	6	0.37	5.80	0.04	2.81	5.29	0.54	1.91	0	0	0	0	0			
15-20m	7	0.23	5.76	0.04	2.44	6.54	0.23	1.94	0	0	0	0	0			
15-20m	8								0	0	0	0	0			
20-25m	-2	0.00						1.49								
20-25m	-1	0.01					0.04	1.67								
20-25m	1	0.00	5.93	0.06	1.20	4.77	0.08	1.88	0	0	0	0	0			
20-25m	2	0.09	3.83	0.05	1.15	3.80			0	0	0	0	0			
20-25m	3	0.05	3.68	0.07	4.80	1.79	0.08	1.65	0	0	0	0	0			
20-25m	4	0.40	5.23	0.08	1.37	5.13	0.07	1.88								
20-25m	5	0.00	4.14	0.03	1.10	4.76	0.12	1.71								
20-25m	6	0.00	5.19	0.04	2.07	5.92	0.04	1.86	0	0	0	0	0			
20-25m	7	0.00	5.44	0.05	2.07	7.04	0.04	1.82	0	0	0	0	0			
20-25m	8								0	0	0	0	0			
25-30m	-2	0.00						1.78								
25-30m	-1	0.01					0.04	1.72								
25-30m	1	0.09	5.36	0.03	1.87	5.25	0.11	1.99	0	0	0	0	0			
25-30m	2	0.01	4.32	0.03	1.33	5.59			0	0	0	0	0			
25-30m	3	0.02	2.57	0.03	4.46	2.97	0.10	1.64								
25-30m	4	0.35	5.29	0.05	0.60	5.54	0.06	1.85								
25-30m	5	0.00	2.84	0.01	1.10	2.62	0.08	1.64								
25-30m	6	0.00	5.54	0.05	2.30	5.99	0.12	1.76								
25-30m	7	0.00	4.59	0.04	2.71	5.74	0.06	1.96	0	0	0	0	0			

8.2. Field Experiment Results

Table 4: Total Iron Content of the Field Samples

Date and Location	Top % Fe (w/w)	Middle % Fe (w/w)	Bottom % Fe (w/w)	Date and Location	Top % Fe (w/w)	Middle % Fe (w/w)	Bottom % Fe (w/w)
1/96 A-1	0.59	0.77	0.33	1/96 M-1	0.29	0.31	0.31
9/96 A-1		1.11		9/96 M-1		0.60	
9/97 A-1		0.96		9/97 M-1		0.29	
1/96 A-2	0.42	0.58	0.45	1/96 M-2	0.23	0.46	0.38
9/96 A-2		1.41		9/96 M-2		0.29	
9/97 A-2		0.18		9/97 M-2		0.32	
1/96 A-3	0.70	0.23	0.14	1/96 M-3	0.63	0.18	0.63
9/96 A-3				9/96 M-3			
9/97 A-3		0.33		9/97 M-3			
1/96 N-1	0.47	0.63	0.20	1/96 F-1	0.51	0.89	0.05
9/96 N-1				9/96 F-1		0.46	
9/97 N-1				9/97 F-1			
1/96 N-2	0.38	0.54	0.78	1/96 F-2	0.60	0.09	0.50
9/96 N-2		0.47		9/96 F-2		0.25	
9/97 N-2		0.30		9/97 F-2		0.09	
1/96 N-3	1.60	0.28	0.65	1/96 F-3	0.03	0.20	0.32
9/96 N-3		0.32		9/96 F-3			
9/97 N-3		0.23		9/97 F-3			
9/96 N-4	0.54	0.85	0.27				
9/97 N-4	0.31	0.29	0.49				
9/96 N-5	0.44	0.45	1.11				
9/97 N-5	0.46	0.50	0.32				

Table 5: Fe/Si ratios of field aquifer sediment samples as determined by SEM/EDS

Date and Location	Top	Middle	Bottom	Date and Location	Top	Middle	Bottom
1/96 A-1		1.38		1/96 M-1			
9/96 A-1				9/96 M-1		2.31	
3/97 A-1		3.23		3/97 M-1		1.68	
9/97 A-1		3.48		9/97 M-1			
1/96 A-2		1.54		1/96 M-2		0.97	
9/96 A-2		2.11		9/96 M-2		1.51	
3/97 A-2		2.49		3/97 M-2		1.88	
9/97 A-2		0.77		9/97 M-2		1.27	
1/96 A-3		1.65		1/96 M-3		0.88	
9/96 A-3				9/96 M-3			
3/97 A-3				3/97 M-3			
9/97 A-3				9/97 M-3			
1/96 N-1		1.22		1/96 F-1			
9/96 N-1				9/96 F-1			
3/97 N-1				3/97 F-1			
9/97 N-1				9/97 F-1			
1/96 N-2		1.31		1/96 F-2		0.92	
9/96 N-2				9/96 F-2		1.48	
3/97 N-2				3/97 F-2		1.30	
9/97 N-2		0.92		9/97 F-2		0.34	
1/96 N-3		1.47		1/96 F-3			
9/96 N-3		1.92		9/96 F-3			
3/97 N-3		1.85		3/97 F-3			
9/97 N-3		1.14		9/97 F-3			
9/96 N-4	2.85	2.90	1.52				
3/97 N-4	4.13	4.82	1.50				
9/97 N-4	0.87	1.01	1.07				
9/96 N-5	1.38	1.56	2.67				
3/97 N-5	4.34	8.20	1.77				
9/97 N-5	2.60	1.70	0.79				

Figure Captions:

Figure 1: Location of field site.

Figure 2: Hydrocarbon Source Zone

Figure 3: Position of wells forming zones near the NAPL source.

Figure 4: Location of sites for both water samples and sediment cores.

Figure 5: XRD pattern over the dominant Fe(III) oxide peak at $\approx 44.6^\circ 2\theta$ for sediments from the near field, middle-depth (MW-10). (a) Comparison of XRD data for as-received and CDB-extracted September 1996 samples. (b) Comparison of samples collected from the same area over time. Note: XRD data are raw, box-car filtered data, calibrated by the $46.405^\circ 2\theta$ quartz peak.

Figure 6: Total Iron from Areas N-2 and N-4 Middle and N-5 Bottom

Figure 7: Representative XRD patterns of sediments after 5 months reaction in the magnetite experiment: (a) Biotic Sample (b) Abiotic Sample

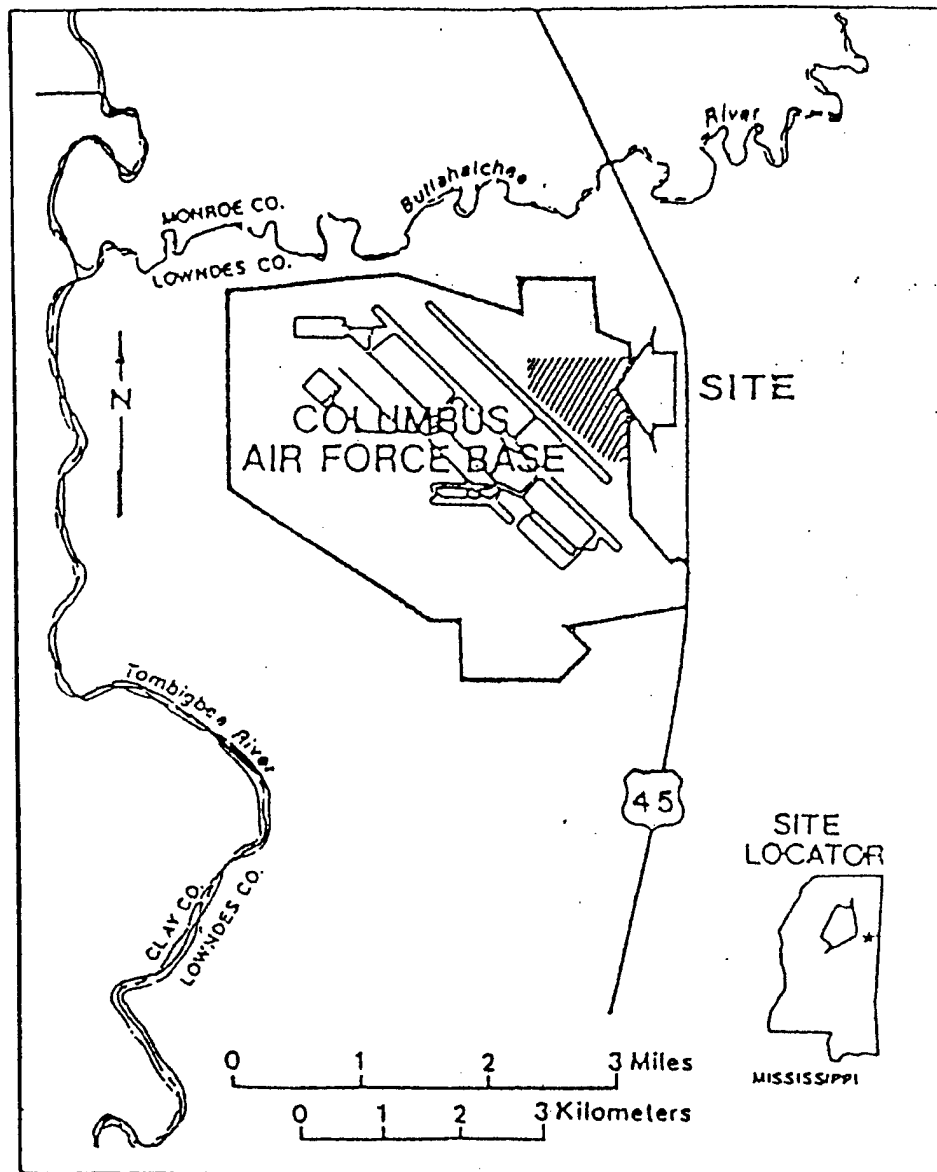


Figure 1

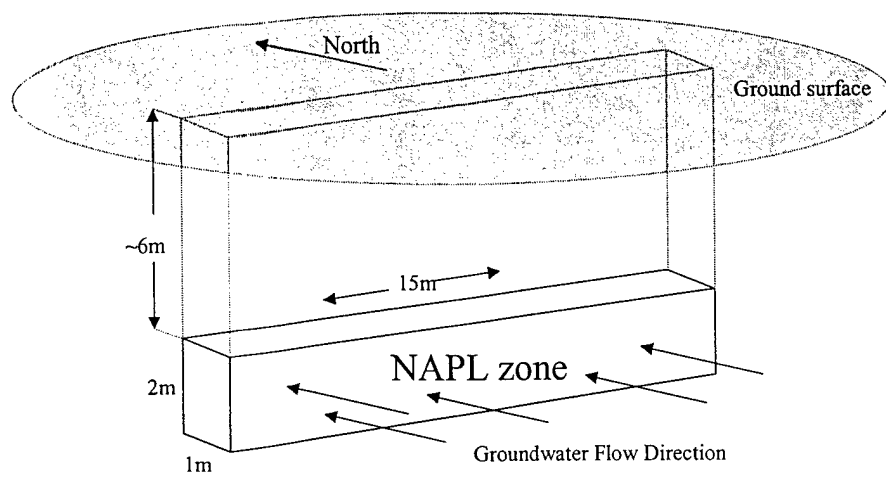


Figure 2

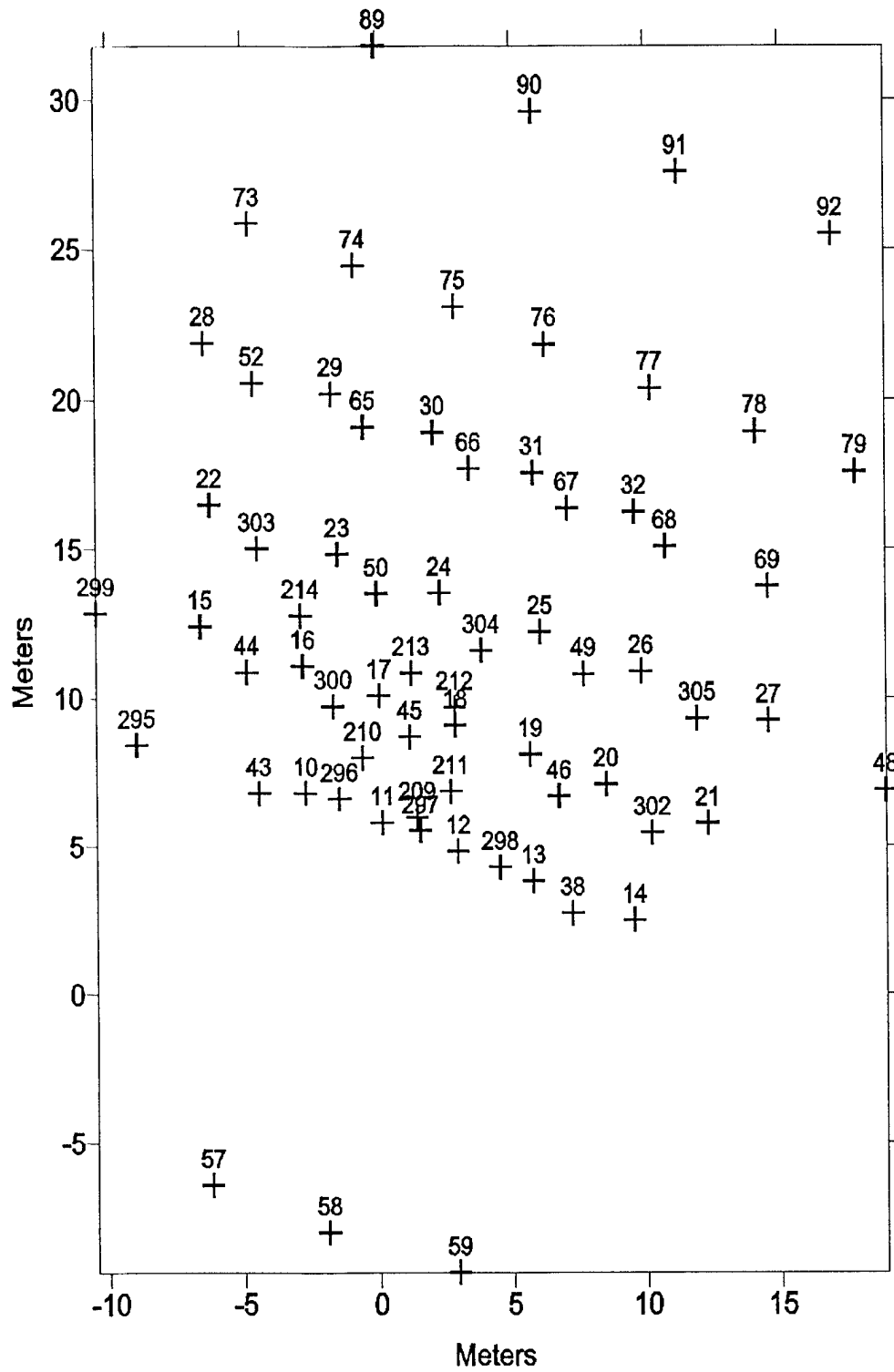


Figure 3

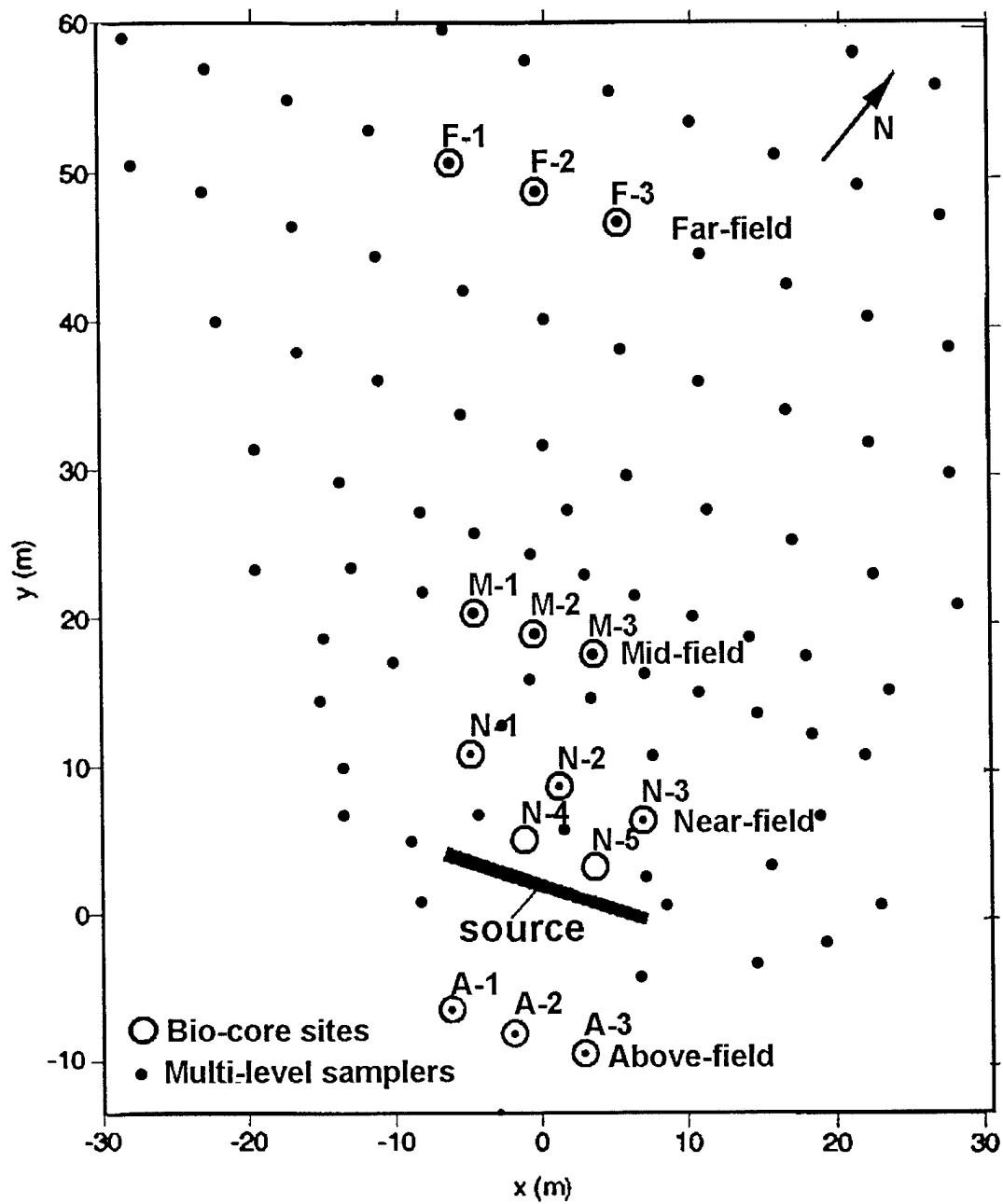


Figure 4

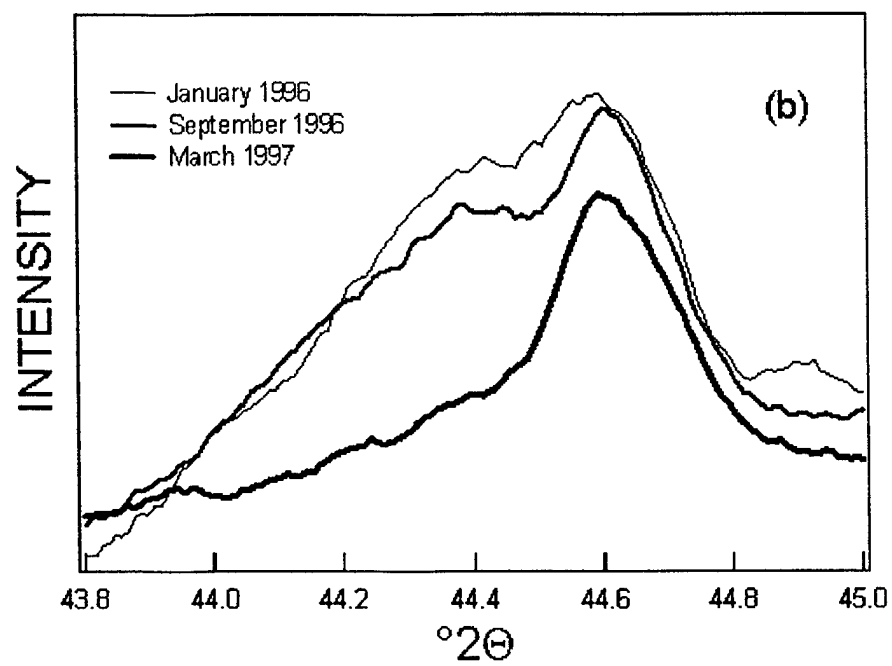
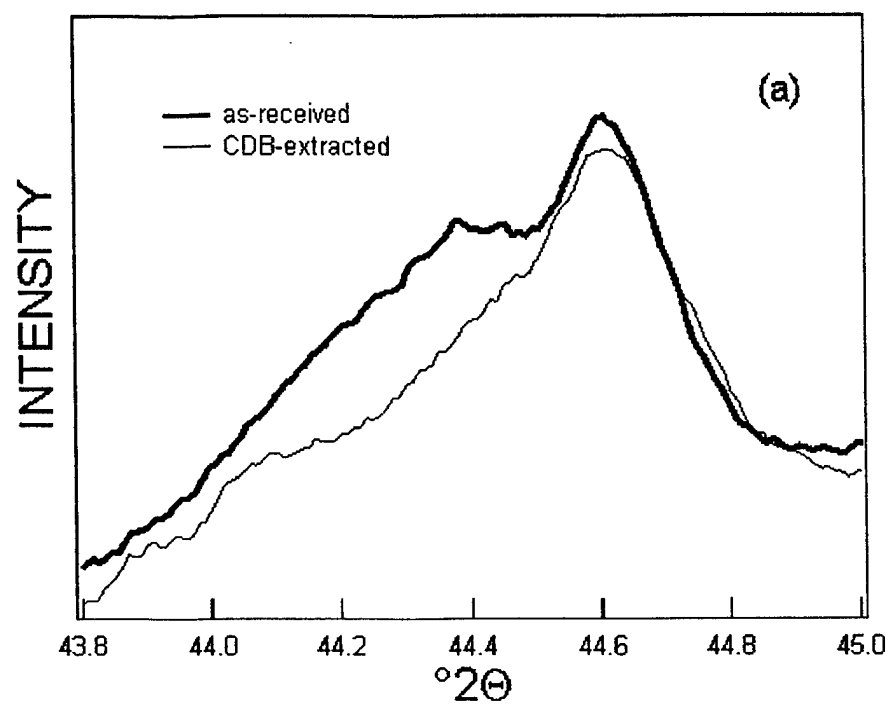


Figure 5

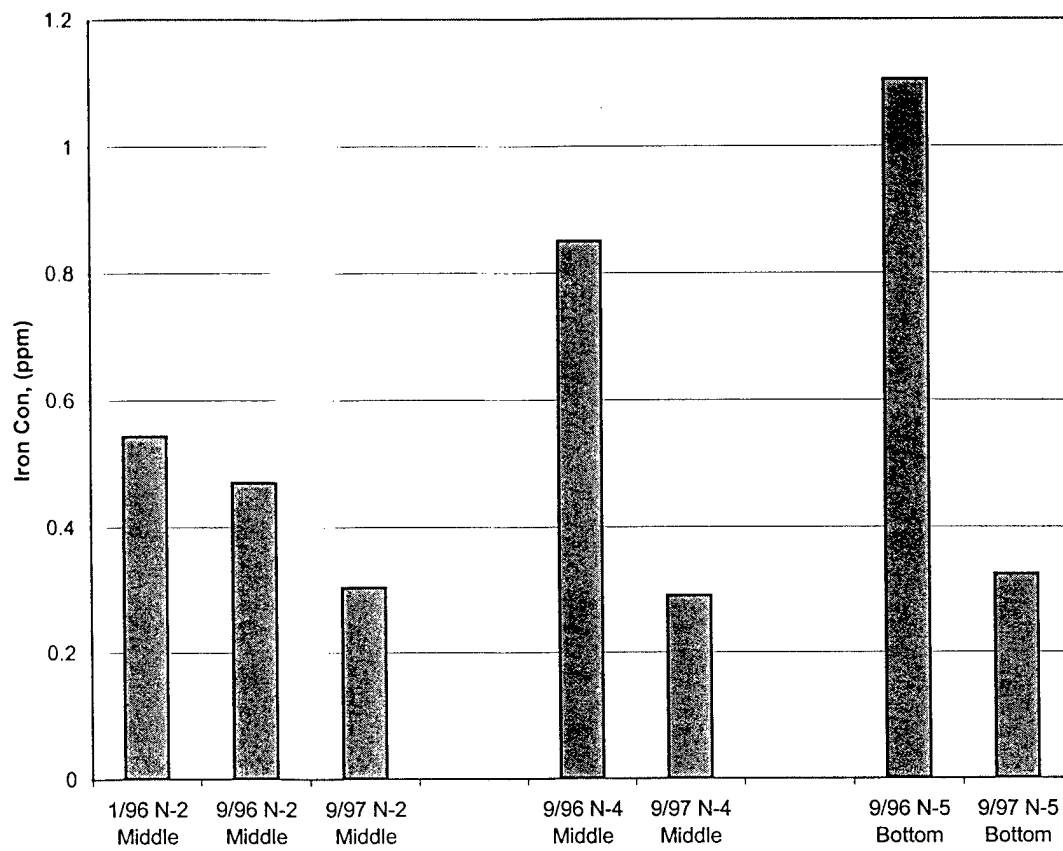


Figure 6

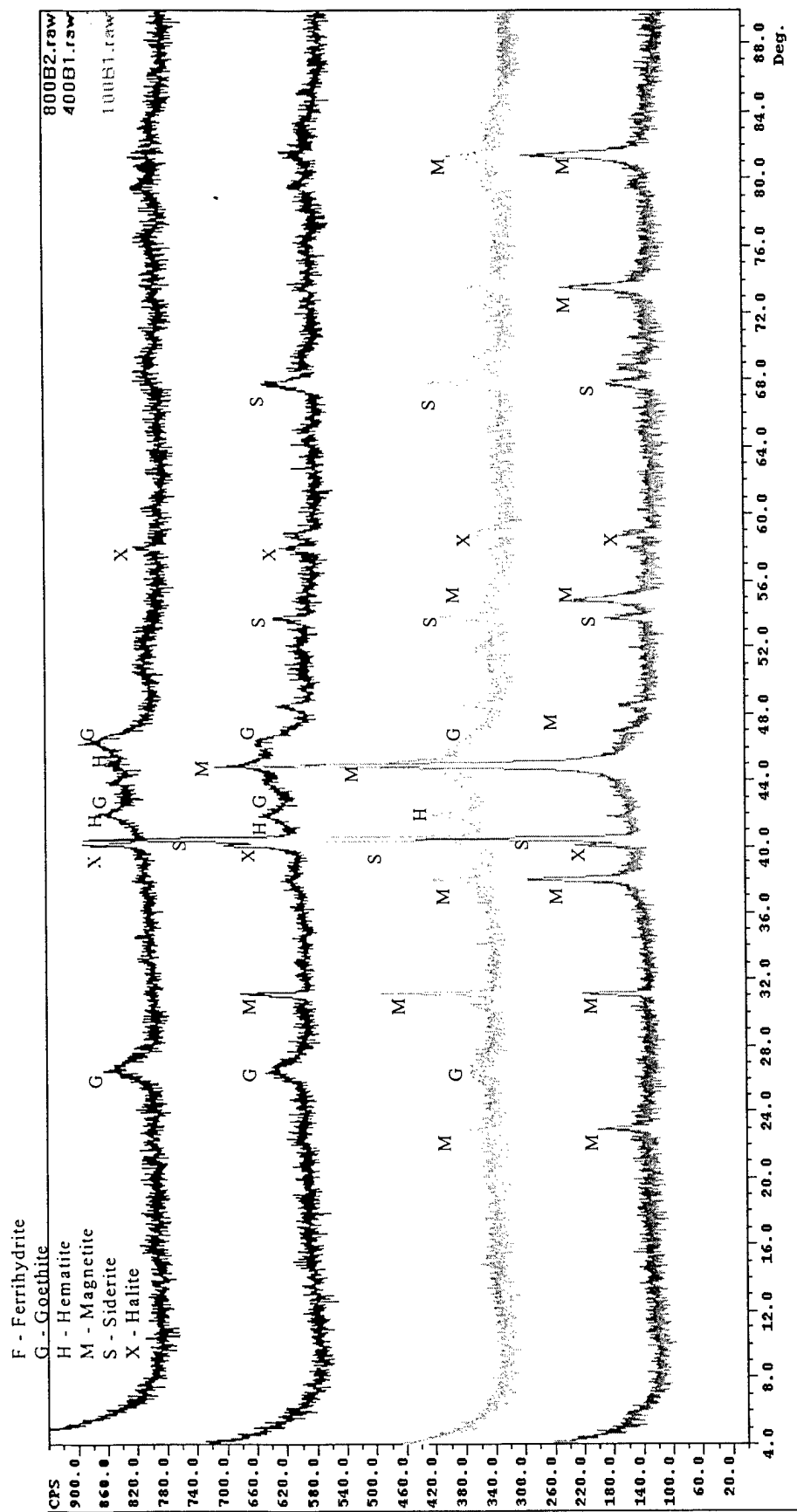


Figure 7a

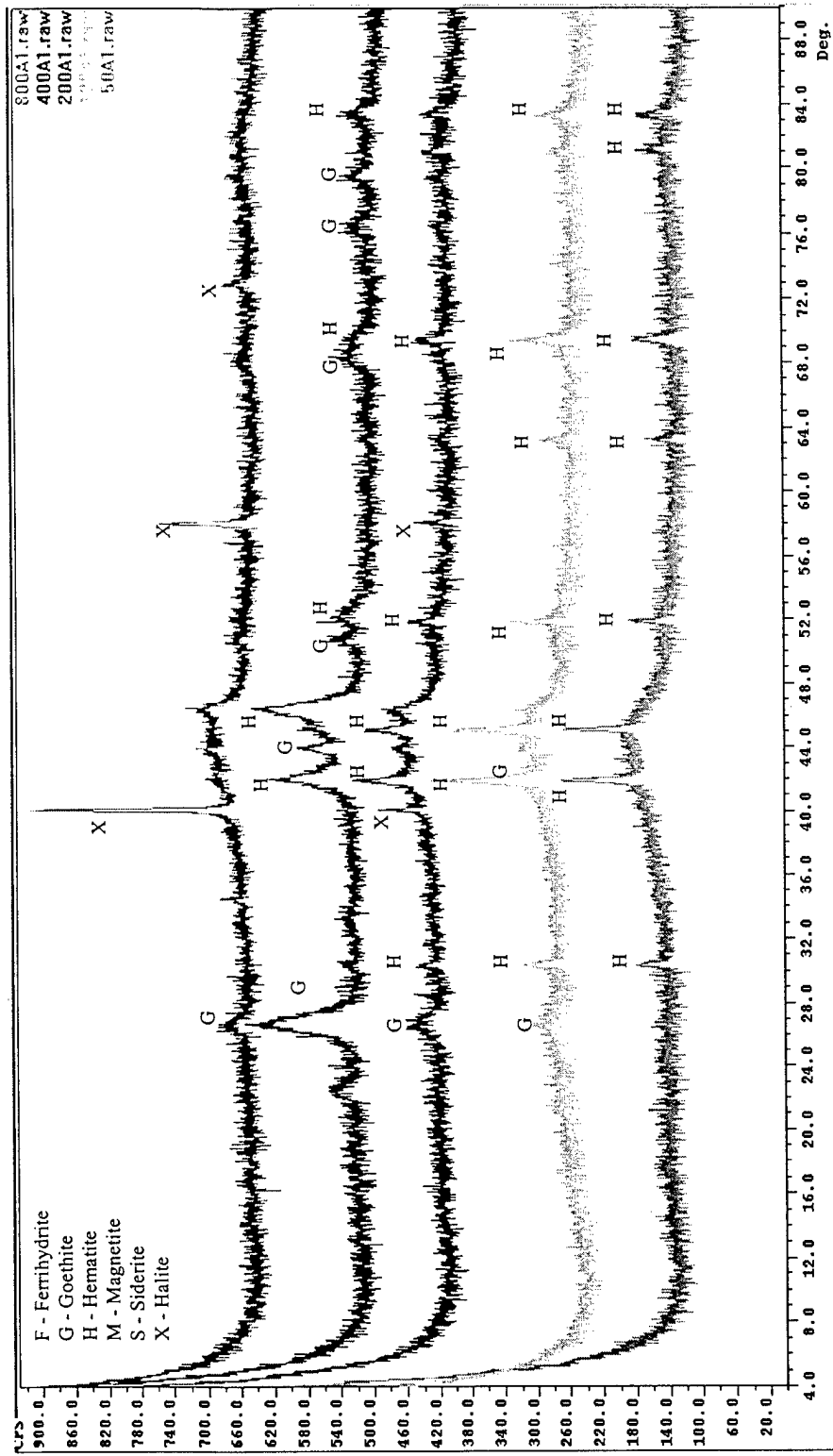


Fig 7b